

## DORSAL TISSUE AFFECTING FACTOR AND COMPOSITIONS

5        This application is a continuation of copending United States  
Patent application Serial No. 08/392,935 filed on March 3, 1995,  
which is the U.S. National Stage of PCT/US93/08326 filed September  
2, 1993, which is a continuation-in-part of U.S. Serial No.  
07/957,401 filed on October 6, 1992, which is a continuation-in-  
10    part of United States Patent Application Serial No. 07/950,410 filed  
on September 23, 1992, which is a continuation-in-part of United  
States Patent Application Serial No. 07/939,954 filed on September  
3, 1992.

### 15    Field of the Invention

The invention generally relates to growth factors and  
neurotrophic factors, and more particularly to a soluble growth  
factor with dorsal growth inducing activity, to complexes including  
the factor, and to DNA or RNA coding sequences for the factor.

20        This invention was made, in part, with government support  
under Grant Contract No. ROI-GM-42341, awarded by the National  
Institutes of Health. The government has certain rights in this  
invention.

### 25    Background of the Invention

Growth factors are substances, such as polypeptide hormones,  
which affect the growth of defined populations of animal cells in  
vivo or in vitro, but which are not nutrient substances. Proteins  
involved in  
30    the growth and differentiation of tissues may promote or inhibit  
growth, and promote or inhibit differentiation, and thus the general  
term "growth factor" includes cytokines and trophic factors. Among  
growth, or neurotrophic factors presently known are those that can  
be classified into the insulin family [insulin, insulin-like growth

EJ915219857US

factors (e.g., IGF-I, IGF-II), mammary stimulating factor (MSF), and nerve growth factor (NGF)]; those classified into the epidermal growth factor family [epidermal growth factor (EGF) and transforming growth factors ( $TGF\alpha$ ,  $TGF\beta$ ,  $TGF\gamma$ )]; those classified  
5 into the platelet-derived growth factor family [platelet-derived growth factor (PDGF), osteosarcoma-derived growth factor (ODGF), and fibroblast growth factor (FGF)]; the neurotrophins [nerve growth factor (NGF), brain derived neurotrophic factors (BDNF) neurotrophins 3, 4, 5, (NT-3, NT-4, NT-5)]; and others [colony  
10 stimulating factor (CSF), T-cell growth factor, tumor angiogenesis factor (TAF), DNA synthesis promoting factor (DSF), tumor-derived growth factors, fibroblast-derived growth factor (FDGF)].

Receptors that affect growth (that is, receptors for growth-associated ligands) are proteins found associated with cell  
15 surfaces that specifically bind their growth factors as ligands. Growth factor receptors are utilized in various clinical and diagnostic applications.

U.S. Patent 4,857,637, issued August 15, 1989, inventors Hammonds et al., describes a method for immunizing an animal  
20 against its growth hormone receptor through use of vaccinating with antibodies in order to stimulate growth of the animals.

U.S. Patent 4,933,294, issued June 12, 1990, inventors Waterfield et al., describes studies of structural alterations of the human EGF receptor and its gene and a relationship in tumorigenesis  
25 for assays and therapies involving the human EGF receptor. For example, such assays can involve detection of structurally altered or abnormally expressed growth factor receptor and the mRNA transcripts and genes which encode them. EGF may have a role in cell

proliferation and differentiation since it induces early eyelid opening and incisor development in new born mice.

U.S. Patent 5,030,576, issued July 9, 1991, inventors Dull et al., describes the role of receptors, such as receptors for growth factors, in designing drugs by the pharmaceutical industry, and discloses use of a receptor hybrid for screening drug purposes, such as in studies of EGF binding domains. U.S. Patent 5,087,616, issued February 11, 1992, inventors Myers and Bichon, describes a method for destroying tumor cells with a composition including a drug conjugate. The conjugate has a growth factor as one moiety and a polymeric carrier with a cytotoxic compound as another moiety. Thus, compositions of the patent are described as binding preferentially to tumor cells bearing EGF-binding receptors (when an EGF growth factor, for example, is used as a first moiety).

U.S. Patent 5,098,833, issued March 24, 1992, inventors Lasky, et al., describes a DNA isolant capable of hybridizing to the epidermal growth factor domain. Expression systems for recombinant production are said to be useful in therapeutic or diagnostic compositions.

A good background review of a neurotrophic factor related to NGF is provided by W092/05254, published April 2, 1992, which also describes state of the art methods of: preparing amino acid sequence variations, site-directed mutagenesis techniques, ligation of coding DNA into a replicable vector for further cloning or for expression, choice of promoters for expression vectors, suitable host cells for expression, particularly mammalian cells, protein purification upon recovery from culture medium as a secreted protein, derivatization with bifunctional agents to cross-link

protein to a support matrix for use with antibodies, entrapment in systems for drug delivery, preparation of therapeutic formulations, and methods of administration. In addition, preparation of polyclonal and monoclonal antibodies are described, such as are  
5 useful in diagnostic assays. These various aspects of isolation, preparation, and applications for a novel neurotrophic factor, as illustrated by the W092/05254 publication, are incorporated herein by reference.

Thus, growth factors, their receptors, and DNA or RNA coding  
10 sequences, therefore, and fragments thereof are useful in a number of therapeutic, clinical, research, diagnostic, and drug design applications.

#### Brief Description of the Drawings

15 Figure 1. Nucleotide sequence (SEQ ID NO:1) for the human noggin gene and deduced amino acid sequence (SEQ ID NO: 2).

Figure 2 (A). Experimental design: competent animal cap(AC) ectoderm was dissected from staged embryos as shown. St10.5 dorsal and ventral AC and ventral marginal zones (VMXZ) also  
20 dissected as shown. Explants were washed once in low Ca/Mg Ringers (LCMR) solution and then placed in treatment medium containing factor diluted in LCMR + 0.5%BSA. Explants cultured to late stages (St20+) were removed from treatment medium 6-16 hours after the start of treatment and placed in LCMR. When explants  
25 reached the desired stage they were either harvested for RNA, or

they were fixed for whole mount *in situ* hybridization or antibody staining.

(B). Neural induction by noggin in the absence of muscle. Lanes 1-3 show specific fragments protected by *N-CAM*,  $\beta$ -tubulin, and XIF-3 probes respectively in whole St24 embryo RNA. Lanes 4-8 show protection by the mixture of these three probes while lanes 9-13 show protection by an actin probe on tRNA(t), St24 embryo RNA (E), and RNA collected from St9 AC treated with 50pM activin (A), 25% of 20 fold concentrated control CHO cell medium (C) or 25% of 20 fold concentrated noggin conditioned CHO cell medium (N). Ubiquitously expressed cytoskeletal actin used as a loading control shows that RNA levels in all treatments are comparable (lanes 11-13).

Figure 3. 12% SDS-PAGE run under reducing conditions.

Proteins were visualized by silver staining. Lane 1 shows molecular size standards. Lanes 2-7 show 0, 0.1, 0.2, 0.5, 1, and 2  $\mu$ g of purified human noggin.

Figure 4 (A). Time course of animal caps treated with purified noggin vs. activin; direct vs indirect neural induction. Animal caps were dissected as shown in Fig. 2A and treated with LCMR + 0.5% BSA (U), a 20% dilution of activin conditioned COS cell medium (A), or 1 $\mu$ g/ml purified human noggin(N). RNA isolated from treated animal caps (lanes 2-13) along with St22 whole embryo RNA (lane1) and tRNA (lane 14) was probed for *N-CAM*,  $\beta$ -tubulin, muscle and cytoskeletal actins, collagen type II, and EF-1a.

(B). Expression of early mesoderm markers in activin but not noggin induced animal caps. Animal caps were dissected from St8 embryos, treated as described in (A), and harvested at St11. Lanes 1

and 2 respectively show *goosecoid* and *Xbra* probe protection by St10.5 whole embryo RNA. Lanes 3-6 show protection by a mix of these two probes. Relative RNA levels are demonstrated by separate *EF-1 $\alpha$*  probe protection.

5 (C). Plasmid directed gastrula stage noggin expression directly induces neural tissue. One cell stage embryos were injected with 20pg of pCSK*lacZ* or pCSK*noggin* into the animal pole. Animal caps from injected embryos were dissected at St8-9 and cultured until St20, when they were harvested for analysis by RNase protection.

10 Figure 5. Responsiveness of dorsal and ventral animal caps to neural induction by noggin. St 10.5 ventral and dorsal animal caps were dissected as shown in Fig. 2. Dorsal and ventral animal caps were treated with activin medium (DA,VA) or 1 $\mu$ g/ml human noggin (DN, VN) and harvested at St26 for RNase protection analysis using *N-*  
15 *CAM*,  $\beta$ -tubulin, and actin as probes.

Figure 6. Dose response of ventral marginal zones and animal caps to human noggin protein. St 10.5 VMZs and St9 animal caps were dissected as shown in Fig. 2A., and treated with 0, 1, 10, 50, 200, and 1000 ug/ml of human noggin (lanes 3-8 and 10-15  
20 respectively). RNA from treated explants and control whole embryos aged to St26 was then analyzed by RNase protection, using the probes *N-CAM*,  $\beta$ -tubulin, actin and collagen type II. In this experiment, muscle induction at the dose of 1ng/ml is stronger than at 10ng/ml, and there is a low level of muscle actin expression in  
25 the uninduced VMZs. This could be due to experimental variability since in repeated experiments we saw muscle induction only at the doses of 50ng/ml and above (data not shown).

Figure 7. *In situ* hybridization and antibody staining. Tailbud embryos stained for *NCAM* showing side and dorsal views (a,b); *NCAM* RNA is only detected in the neural tube, and not the somites. For comparison, somites of a tailbud embryo stain for muscle actin, dorsal view (c). Neural specific 6F11 antibody staining at St30 (d-f). Some cement gland pigment remained in these embryos after bleaching as seen in (d), however this pigment is distinct from antibody staining. The inner mass of staining in the noggin treated animal caps is due to the 6F11 antibody detection. Cement gland specific *XAG-1* transcripts detected at St23 (g-i), and anterior brain *otxA* transcripts detected at St35 (j-l) in whole embryos at (d,g,j), human noggin treated (1µg/ml) animal caps (e,h,k), and untreated animal caps (f,i,l).

Figure 8. Reverse phase HPLC profile of two refolded isoforms of noggin. The refolded noggin solution was applied onto a Brownlee Aquapore AX-300, 0.46 x 22 cmHPLC column at a flow rate of 1 ml/min. The column was equilibrated with solvent A containing 0.1% TFA in water. Solvent B was 0.1% TRA in acetonitrile. The column was developed according to the following protocol: a) 2 min isocratically at 95% of solvent A-5% of solvent B; 60 min linear gradient to 65% of solvent B and 35% of solvent A. Correctly refolded noggin elutes earlier at 44%-46% solvent B.

Figure 9. Reverse-phase HPLC chromatography characterization of recombinant noggin refolded and purified from *E. coli*. Conditions as in the legend to Fig. 8.

Figure 10. Recombinant noggin produced in *E. coli* and in insect cells analyzed by 12.5% SDS-PAGE. Lanes H, L: High and low molecular weight markers of the indicated size, respectively. Lanes

- 1,2: Recombinant noggin produced in *E. coli* and in insect cells respectively, treated with 2-mercaptoethanol before electrophoresis. The slower mobility of noggin from insect cells corresponds to the size increase that would occur due to N-linked glycosylation at the single consensus site. Lanes 2,3: Recombinant  
 5 noggin produced in *E. coli* and in insect cells respectively, not treated with 2-mercaptoethanol before electrophoresis.

Figure 11. Circular dichroism spectra of recombinant noggin produced in *E. coli* (--), and in insect cells (-).

- 10 Figure 12. Ventral marginal zone assay showing induction of muscle actin mRNA after exposure to human noggin (0.01, 0.05, 0.2 µg/ml) produced in baculovirus, a mock transfected culture of baculovirus (0.02, 1 µg/ml) or human noggin produced in *E.coli* (0.1, 0.5, 2, or 10 µg/ml).

- 15 Figure 13. Nucleotide sequence (SEQ ID NO:25) for the mouse noggin gene and deduced amino acid sequence (SEQ ID NO:26).

#### Summary of the Invention

- 20 In one aspect of the present invention a peptide that can be in substantially purified form is characterized by one or more of the following, highly conserved amino acid sequences:

- QMWLWSQTFCPVLY (SEQ ID NO:3);  
 RFWPRYVKVGSC (SEQ ID NO:4);  
 25 SKRSCSVPEGMVCK (SEQ ID NO:5);  
 LRWRCQRR (SEQ ID NO:6); and,  
 ISECKCSC (SEQ ID NO:7).



Peptides of the invention induce dorsal growth in vertebrates and can be prepared in soluble, physiologically active form for a number of therapeutic, clinical, and diagnostic applications.

In a preferred embodiment, human noggin protein as set forth  
5 in Figure 1 (SEQ ID NO: 2) is prepared for use in therapeutic, clinical and diagnostic applications.

In another aspect of the present invention an oligonucleotide, such as cDNA, is provided having substantial similarity to (or being the same as) SEQ ID NO:8 (deduced amino acid sequence, SEQ ID NO:  
10 9), SEQ ID NO: 10 (deduced amino acid sequence, SEQ ID NO: 11), or SEQ ID NO:1. This oligonucleotide can be single or double stranded, be formed of DNA or RNA bases, and can be in the antisense direction with respect to SEQ ID NOS: 8, 10 or 1. SEQ ID NO: 8, SEQ ID NO: 10 and SEQ ID NO:1 each code for a functional polypeptide that we have  
15 designated "noggin," which is capable of inducing dorsal development in vertebrates when expressed.

Noggin or fragments thereof (which also may be synthesized by in vitro methods) may be fused (by recombinant expression or in vitro covalent methods) to an immunogenic polypeptide and this, in  
20 turn, may be used to immunize an animal in order to raise antibodies against a noggin epitope. Anti-noggin is recoverable from the serum of immunized animals. Alternatively, monoclonal antibodies may be prepared from cells to the immunized animal in conventional fashion. Antibodies identified by routine screening will bind to  
25 noggin but will not substantially cross-react with "wnt" or other growth factors. Immobilized anti-noggin antibodies are useful particularly in the diagnosis (in vitro or in vivo) or purification of noggin.

Substitutional, deletional, or insertional mutants of noggin may be prepared by in vitro or recombinant methods and screened for immuno-crossreactivity with noggin and for noggin antagonist or agonist activity.

- 5           Noggin also may be derivatized in vitro in order to prepare immobilized noggin and labelled noggin, particularly for purposes of diagnosis of noggin or its antibodies, or for affinity purification of noggin antibodies.

10           The present invention further provides for expression of biologically active noggin molecules in prokaryotic and eukaryotic expression systems.

15           The present invention further provides for the production of noggin in quantities sufficient for therapeutic and diagnostic applications. Likewise, anti-noggin antibodies may be utilized in therapeutic and diagnostic applications. For most purposes, it is preferable to use noggin genes or gene products from the same species for therapeutic or diagnostic purposes, although cross-species utility of noggin may be useful in specific embodiments of the invention.

20           In additional embodiments, the noggin nucleic acids, proteins, and peptides of the invention may be used to induce neural tissue formation in mammals.

#### Description of the Preferred Embodiments

25           We have discovered a structurally unique growth factor that is readily available in substantially pure, soluble form. We have named the inventive polypeptide "noggin." This newly isolated neurotrophic factor induces dorsal development in vertebrates.

An earlier described family of proteins that also induces dorsal development are the "wnt" proteins. These, however, in contrast to noggin remain tenaciously bound to cell surfaces. Our initial work with noggin has been in *Xenopus* embryos; however, 5 noggin is highly conserved among vertebrates, as our work with mouse noggin has demonstrated. The prior known FGF growth factor family is also known to be involved in early embryonic induction, but both the FGF proteins and their receptors are distinctly different from noggin. Noggin modifies the actions of FGF (and also activin), 10 for example by potentiating growth, and is thus particularly suggested in therapeutic compositions for use in combination with other growth factors (as therapeutic adjuvants), such as to modify or potentiate their effects.

We have cloned cDNA for noggin. The noggin cDNA contains a 15 single reading frame encoding a 26 kDa protein with a hydrophobic amino-terminal sequence. Noggin is secreted. Noggin's cDNA encodes the protein as a 26 kDa protein, but we have determined that noggin is secreted in vivo, apparently as a dimeric glycoprotein with a starting apparent molecular weight of about 33 kDa (as the wild- 20 type subunit). When not glycosylated, the monomeric unit has an apparent molecular weight on SDS PAGE of about 25-30 kDa.

We have cloned the gene for human noggin (Figure 1; SEQ ID NO: 1). The sequence codes for a protein which has noggin activity (SEQ ID NO: 2). The carboxy terminal region of noggin shows homology to a 25 Kunitz-type protease inhibitor, indicating that noggin protein, or fragments thereof, may exhibit activities of a protease inhibitor.

We have been able to express biologically active noggin in both eukaryotic and prokaryotic host cells. Two expression systems

we have successfully used to express biologically active noggin have been mammalian cell lines (COS and mouse 293). A third expression system is injection of synthetic mRNA into *Xenopus* oocytes. In addition, we have successfully expressed biologically active human  
 5 noggin in a prokaryotic system, *E. coli*, and in baculovirus.

Expression in these several different systems also illustrates the high degree of conservation for noggin. We have found, for example, substantial sequence similarity between frog noggin and mouse noggin with a number of completely conserved stretches.  
 10 Thus, the following amino acid sequences represent completely conserved portions as between frog noggin and mouse noggin:

QMWLWSQTFCPVLY (SEQ ID NO:3);  
 RFWPRYVKVGSC (SEQ ID NO:4);  
 SKRSCSVPEGMVCK (SEQ ID NO:5);  
 15 LRWRCQRR (SEQ ID NO:6); and,  
 ISECKCSC (SEQ ID NO:7).

There is about 87% overall conservation between the mouse and frog sequences, and we have also observed a unique cysteine distribution between the two.

20 Noggin nucleic acids, or oligonucleotides, encode a noggin polypeptide or hybridize to such DNA and remain stably bound to it under stringent conditions and are greater than about 10 bases in length; provided, however, that such hybridizing nucleic acid is novel and unobvious over any prior art nucleic acid including that  
 25 which encodes or is complementary to nucleic acid encoding other growth factors.

By "stringent conditions" we mean are those which (1) employ low ionic strength and high temperature for washing, for example,

0.15 M NaCl/0.015 M sodium citrate/0.1% NaDodSo<sub>4</sub> at 50°C, or (2) use during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C.

By "substantial similarity," when we are referring to a nucleotide sequence, is meant cross hybridization of sequences under conditions of moderate stringency using a probe greater than 100 nucleotides long at 30°C in a standard buffer (Wahl et al., PNAS, 76, 3683) and washes at 37°C in 300 mM NaCl, 30 mM sodium citrate, 0.2% SDS at pH 7. Alternatively, one is able to isolate, by polymerase chain reaction, a fragment of DNA coding for noggin or noggin family members when using primers of degenerate sequence that encode those SEQ ID NOS:3-7.

By "substantial similarity" when reference is made to proteins is that noggin from different species, or noggin family members within a species, will preserve the positions of cysteine residues in at least 80% of positions throughout the protein. Like the neurotrophin family, the sequence of the mature form of noggin and noggin related polypeptides will be identical in at least 40% of positions. Substantial similarity at the protein level includes an ability of a subject protein to compete with noggin for binding to receptors and some (but not all) monoclonal antibodies raised against noggin epitopes.

The cloned cDNA for noggin (derived from frog) is designated herein as SEQ ID NO: 8, partial sequence from mouse as SEQ ID NO: 10 or full sequence of mouse noggin as shown in Figure 13 (SEQ ID

NO: 25). The human sequence is designated herein as SEQ ID NO: 1.

We have used RNA transcripts from the SEQ ID NO: 8 clone to rescue embryos and return them to substantially normal development when the noggin RNA is injected into ventralized embryos. In high doses

5 this results in excessive head development and it is for this reason we named the protein "noggin." In northern blot analysis the noggin cDNA hybridizes to two mRNAs that are expressed both maternally and zygotically.

When using nucleotide sequences coding for part or all of  
10 noggin in accordance with this invention, the length of the sequence should be at least sufficient in size to be capable of hybridizing with endogenous mRNA for the vertebrate's own noggin. Typically, sufficient sequence size (for example, for use as diagnostic probes) will be about 15 consecutive bases (DNA or RNA). In some diagnostic  
15 and therapeutic applications, one may wish to use nucleotide noggin coding sequences (analogous to all or a portion of SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 25 or SEQ ID NO:1) in the anti-sense direction with respect to either SEQ ID NOS: 8, 10, 25, or 1.

We suggest as a few preferred primers for amplifying noggin  
20 from other species (e.g. human):

5' Primer 1 SEQ ID NO: 12

C A A/G A C N T T C/T T G C/T C C N G T N

5' Primer 2 SEQ ID NO: 13

T T C/T T G G C C N C/A G N T A C/T G T N A A A/G G T N G G

25 5' Primer 3 SEQ ID NO: 14

C C N G A A/G G G N A T G G T N T G

3' Primer 1 SEQ ID NO: 15

C A N C/G T/A A/G C A C/T T T A/G C A C/T T C

3' Primer 2 SEQ ID NO: 16

C A N A C C A T N C C C / T T C N G G

3' Primer 3 SEQ ID NO: 17

C G / T N C G / T T / C T G G / A C A N C G / T C C A

5

where N represents a mixture of all four nucleotides and mixtures of two nucleotides are represented by alternates (e.g. A/G).

Although noggin transcript is not localized in the oocyte and cleavage stage embryo, zygotic transcripts are initially restricted to the presumptive dorsal mesoderm, and reach their highest levels at the gastrula stage in the dorsal lip of the blastopore (Spemann's organizer). In the neurula, noggin is transcribed in the notochord and prechordal mesoderm.

Without being bound by theory, we have formulated hypotheses about the embryological effects of noggin based on where it is expressed, and on the effects of RNA injection in embryos. Since noggin is expressed in the Spemann organizer, we believe noggin to be a mediator of the effects of the Spemann organizer, namely neural induction and dorsalization of the mesoderm. We have shown that noggin is able to directly induce neural tissue formation. Since noggin is expressed in the notochord and head mesoderm, we believe noggin to influence either the dorsal-ventral pattern or anterior-posterior pattern of the neural plate. Since noggin is expressed in the branchial arch neural crest, we believe it may therefore influence whether neural crest cells deposit cartilage and also to influence later branchial arch growth and remodelling. Noggin is expressed in the tail fin neural crest, and since neural crest is

required for growth of the fin, noggin may act as a growth factor for epidermis or mesenchyme.

Although much of our experimental work has involved rescue of embryonic development, because expression in the notochord persists in the growing tail bud and a discontinuous line of stained cells (indicating expression of noggin initiated at new sites) runs the length of the roof plate of the neural tube (and is also apparent in the head mesoderm), we believe noggin is expressed as an adult cell function also.

10 A number of applications for noggin are suggested from its properties.

The noggin cDNA should be useful as a diagnostic tool (such as through use of antibodies in assays for proteins in cell lines or use of oligonucleotides as primers in a PCR test to amplify those with sequence similarities to the oligonucleotide primer, and to see how much noggin is present, e.g. primers such as 5' Primers 1-3 and 3' Primers 1-3).

Because noggin has a pattern of expression that suggests it is used to regulate cartilage production in the embryonic head, clinical uses to regulate cartilage and bone growth are suggested for noggin in therapeutic compositions and particularly in combination with other growth factors due to a property of noggin to potentiate at least some growth factors. Since neural crest cells are required for the tadpole fin to grow, noggin seems to be a growth factor for the tissue matrix and epidermis and should prove useful, for example, in wound healing compositions.

Noggin, of course, provides the key to isolate its receptor. Since many receptors mutate to cellular oncogenes, the noggin



receptor should prove useful as a diagnostic probe for certain tumor types. Thus, when one views noggin as ligand in complexes, then complexes in accordance with the invention include antibody bound to noggin, antibody bound to peptides derived from noggin, noggin bound to its receptor, or peptides derived from noggin bound to its receptor. Mutant forms of noggin, which are either more potent agonists or antagonists, are believed to be clinically useful. Such complexes of noggin and its binding protein partners will find uses in a number of applications.

Practice of this invention includes use of an oligonucleotide construct comprising a sequence coding for noggin and for a promoter sequence operatively linked to noggin in a mammalian, bacterial or a viral expression vector. Expression and cloning vectors contain a nucleotide sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomes, and includes origins of replication or autonomously replicating sequences. The well-known plasmid pBR322 is suitable for most gram negative bacteria, the 2 $\mu$  plasmid origin for yeast and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. Typically, this is a gene that encodes a protein necessary for the survival or growth of a host cell transformed with the vector. The presence of this gene ensures that any host cell which deletes the vector will not obtain an advantage in growth or reproduction over transformed hosts.

Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, (b) complement auxotrophic deficiencies.

- 5           Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR) or thymidine kinase. Such markers enable the identification of cells which were competent to take up the noggin nucleic acid. The mammalian cell transformants are placed under selection pressure in which only the transformants  
10 are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed. Amplification is the process by which genes in greater demand for  
15 the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of noggin can therefore be synthesized from the amplified DNA.

- For example, cells transformed with the DHFR selection gene  
20 are first identified by culturing all of the transformants in a culture medium which contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Nat.  
25 Acad. Sci., 77, 4216 (1980). The transformed cells then are exposed to increased levels of Mtx. This leads to the synthesis of multiple copies of the DHFR gene and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding

noggin. Alternatively, host cells transformed by an expression vector comprising DNA sequences encoding noggin and aminoglycoside 3' phosphotransferase (APH) protein can be selected by cell growth in medium containing an aminoglycosidic antibiotic such as kanamycin or neomycin or G418. Because eukarotic cells do not normally express an endogenous APH activity, genes encoding APH protein, commonly referred to as neo resistant genes, may be used as dominant selectable markers in a wide range of eukaryotic host cells, by which cells transformed by the vector can readily be identified.

Expression vectors, unlike cloning vectors, should contain a promoter which is recognized by the host organism and is operably linked to the noggin nucleic acid. Promoters are untranslated sequences located upstream from the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of nucleic acid under their control. They typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters can be operably linked to noggin encoding DNA by removing them from their gene of origin by restriction enzyme digestion, followed by insertion 5' to the start codon for noggin.

Nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein which participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist then synthetic oligonucleotide adapters or linkers are used in accord with conventional practice.

Transcription of noggin-encoding DNA in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma, cytomegalovirus, adenovirus, retroviruses, hepatitis-B virus, and most preferably Simian Virus 40 (SV40), or from heterologous mammalian promoters, e.g. the actin promoter. Of course, promoters from the host cell or related species also are useful herein.

In particular embodiments of the invention expression of noggin in *E. coli* is preferably performed using vectors which comprise the following: a lac UV5 promoter which may be controlled by the lactose operon repressor; a strong ribosome binding site, for example, the ribosome binding site of bacteriophage T7; a mutation in the replication control region of the plasmid which may increase

copy number; and a mutation which limits the expression of the antibiotic resistance protein.

In a preferred embodiment, noggin is expressed in a high copy number kanamycin resistant pBR322-derived plasmid under the control of a lac UV5 promoter. In an additional preferred embodiment, noggin is expressed in baculovirus under the control of the polyhedrin promoter of Autographa californica Multiple Nuclear Polyhedrosis virus in insect host cells.

Noggin is believed to find use as an agent for enhancing the survival or inducing the growth of nerve and muscle cells. It, therefore, is useful in the therapy of congenital conditions or degenerative disorders of the nervous system ("neurodegenerative diseases"), including such diseases as Alzheimer's disease, Parkinson's disease, Huntington's chorea, ALS, peripheral neuropathies, and other conditions characterized by necrosis or loss of neurons, whether central, peripheral, or motoneurons. In addition, it may be useful for treating damaged nerve cells, e.g., nerves damaged by traumatic conditions such as burns and wounds, diabetes, kidney dysfunction, and the toxic effects of chemotherapeutics used to treat cancer and AIDS. It also is useful as a component of culture media for use in culturing nerve cells in vitro.

The capacity of noggin to induce neural tissue may be useful in diseases where neural tissue is formed improperly or incompletely during development. Thus, noggin and the noggin gene are also useful in treating congenital malformations such as anencephaly, or the loss of cerebral hemispheres which results from failure of closure of the anterior neural tube during development.

Practice of this invention includes preparation and uses of a diagnostic or therapeutic agent comprising a nucleotide sequence of at least about 15 DNA or RNA bases analogous to all or a portion of either SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO:25, or SEQ ID NO: 1 or of the nucleic acid sequences contained in bacteriophages, hnogλ-9 or hnogλ-10. That is, noggin preparations are useful as standards in assays for noggin and in competitive-type receptor binding assays when labelled with radioiodine, enzymes, fluorophores, spin labels, and the like. Therapeutic formulations of noggin are prepared for storage by mixing noggin having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers, in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins. Other components can include glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

Noggin may be used according to the invention as described supra. The concentration of the active ingredient used in the formulation will depend upon the effective dose required and the mode of administration used. The dose used should be sufficient to

achieve circulating plasma concentrations of active ingredient that are efficacious. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

By referring to noggin, the present invention also contemplates  
5 the use of fragments, derivatives, agonists or antagonists of noggin molecules.

Noggin may be administered in any pharmacologically acceptable carrier. The administration route may be any mode of administration known in the art, including but not limited to  
10 intravenously, intrathecally, subcutaneously, by injection into involved tissue, intraarterially, intranasally, orally, or via an implanted device. The present invention provides for pharmaceutical compositions comprising noggin in a pharmacologically acceptable carrier.

15 Administration may result in the distribution of noggin throughout the body or in a localized area. For example, in some conditions which involve distant regions of the nervous system, intravenous or intrathecal administration of noggin may be desirable. Alternatively, and not by way of limitation, when  
20 localized regions of the nervous system are involved, local administration may be desirable. In such situations, an implant containing noggin may be placed in or near the lesioned area. Suitable implants include, but are not limited to, gelfoam, wax, or microparticle-based implants.

25 Depending upon the mode of administration, the active ingredient may be formulated in a liquid carrier such as saline, incorporated into liposomes, microcapsules, polymer or wax-based

and controlled release preparations, or formulated into tablet, pill or capsule forms.

Inventive complexes comprise a ligand characterized by one or more of the SEQ ID NOS:3-7. The ligand can be bound to a protein, such as antibody. Such antibodies can be polyclonal or monoclonal. Polyclonal antibodies to noggin generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of noggin and an adjuvant. It may be useful to conjugate noggin or a fragment containing the target amino acid sequence to a protein which is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxy-succinimide (through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N} = \text{C} = \text{NR}$ .

Animals can be immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1  $\mu\text{g}$  of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally in multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later animals are bled and the serum is assayed for anti-noggin titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same noggin polypeptide, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein



fusions. Also, aggregating agents such as alum are used to enhance the immune response.

Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional  
5 fashion, e.g. by fusion with myeloma cells or by EB virus transformation and screening for clones expressing the desired antibody.

In a preferred embodiment, a rat monoclonal antibody such as RP57-16, prepared after immunization of a rat with recombinant  
10 human noggin, reacts specifically with both *Xenopus* and human noggin, but not with the neurotrophins BDNF, NT-3 and NT-4.

Noggin antibodies are useful in diagnostic assays for noggin or its antibodies. In one embodiment of a receptor binding assay, an antibody composition which binds to all of a selected plurality of  
15 members of the noggin family is immobilized on an insoluble matrix, the test sample is contacted with the immobilized antibody composition in order to adsorb all noggin family members, and then the immobilized family members are contacted with a plurality of antibodies specific for each member, each of the antibodies being  
20 individually identifiable as specific for a predetermined family member, as by unique labels such as discrete fluorophores or the like. By determining the presence and/or amount of each unique label, the relative proportion and amount of each family member can be determined.

25 Noggin antibodies also are useful for the affinity purification of noggin from recombinant cell culture or natural sources. Noggin antibodies that do not detectably cross-react with other growth

factors can be used to purify noggin free from these other family members.

Aspects of the invention will now be illustrated by the following examples.

5

## EXPERIMENTAL PROCEDURES

### Production of Xenopus embryos

Xenopus embryos were prepared by the protocol described by  
10 Condie and Harland (Development, 101, 93-105, 1987). Embryos were staged according to the table of Nieuwkoop and Faber ("Normal Table of Xenopus laevis"(Daubin), Amsterdam: North Holland, 1967).  
Ventralized embryos were produced by irradiation with a Statalinker (Stratagene), and dorsalized embryos were produced by treatment  
15 with LiCl as described by us in our paper on certain "wnt" proteins (designated "Xwnt-8"), Smith and Harland, Cell, Vol. 67, pp. 753-765 (1991) (incorporated by reference and occasionally referred to hereinafter as "S&H, supra").

20

### EXAMPLE 1

#### Isolation and Sequencing of Noggin cDNA

The construction of the size-selected plasmid cDNA library from stage 11 LiCl-treated embryos was as follows. Sixty  
25 micrograms of poly(A) RNA from stage 11 LiCl-treated embryos was size fractionated on a 10% to 30% sucrose gradient in the presence of methylmercuric hydroxide. First strand cDNA was synthesized from 2  $\mu$ g of the size-fractionated poly(A) RNAs primed with

oligo(dT) oligonucleotide containing the recognition site for NotI. After synthesis of the second strand, cDNAs were treated with EcoRI methylase, ligated with EcoRI linkers, digested with EcoRI and NotI, and finally ligated to 125 ng of modified pGEM-5Zf(-)

- 5 (Promega). The pGEM-5Zf(-) used here was modified by the addition of an oligonucleotide into the NsiI site to create an EcoRI site. The vector was not treated with alkaline phosphatase, but the excised polylinker sequence was removed on a sepharose 4BCL column. The ligated products were used to transform XL-I Blue cells
- 10 (Stratagene), and plated to give 100,000 colonies per 10 cm plate. Plasmid DNAs were isolated from plate cultures by the alkaline-lysis/polyethylene glycol precipitation protocol.

- Dorsalizing activity in the library was assayed by injecting RNA transcripts made from pooled plasmid DNA. Single clones were
- 15 isolated by a process of sib selection. In this procedure the plasmid library was replated on 12 plates with 10-fold fewer colonies per plate. RNA was synthesized from pooled plasmid DNAs isolated from each plate and tested for dorsalizing activity by injection into UV-ventralized embryos. Those pools with dorsalizing activity were
- 20 replated and screened as described above. This process was repeated until single clones were isolated.

*In vitro* RNA synthesis, injection assay for dorsal axis rescue and sib-selections were also done, as described by us in S&H, supra.

- The nucleotide sequence of both strands of the isolated noggin
- 25 cDNA clone was determined by the dideoxy termination method using modified T7 DNA polymerase (US Biochem). Deletions were prepared in sequencing templates by both restriction enzyme and

exonuclease III digestion (Henikoff, Meth. Enzymol, 155, 156-165, 1987).

#### In vitro translation

- 5 One-half  $\mu$ g of in vitro synthesized noggin, Xwnt-8, and goosecoid mRNAs were translated in a nuclease treated rabbit reticulocyte lysate (Promega) with added  $^{35}$ S-methionine according to the manufacturer's instructions. The translation products were visualized by SDS-polyacrylamide gel electrophoresis (12% gels)  
10 followed by fluorography. Noggin protein had the molecular weight predicted by the open reading frame.

#### RNA Isolation and Analysis

- Total RNA was isolated from embryos and oocytes by a small  
15 scale protocol as described by Condie and Harland, supra. Dorsal lips were dissected from 30 unfixed stage 10.5 embryos and pooled for total RNA preparation. Samples containing either the total RNA equivalent of 2.5 embryos or approximately 2  $\mu$ g of poly A+ RNA were analyzed by northern blotting. Random primed DNA probes were  
20 prepared from a 1,323 bp fragment of noggin cDNA from the EcoRI site at nucleotide -83 to an EcoRV site that lies in the vector immediately 3' to the end of the cDNA.

- RNAse protection assays were done using a protocol as detailed by Melton et al. (Nuc. Acids Res., 12, 7035-7056, 1984)  
25 with minor modifications (C. Kintner, Salk Institute, La Jolla, California). A noggin cDNA exonuclease III deletion clone, illustrated by SEQ ID NO: 8 but having a deletion from the 3' end to nucleotide 383, was used as a template for synthesizing RNA

probes. The template DNA was linearized by EcoRI restriction enzyme digestion and a 463 base antisense RNA incorporating  $^{32}\text{P}$  was synthesized with T7 RNA polymerase. A 387 base antisense EFl $\alpha$  RNA probe was used as a control for amount of RNA per sample.

- 5 Probes were gel purified prior to use.

#### In situ hybridization

- After fixation and storage, the embryos were checked to ensure the blastocoel and archenteron were punctured. Care was  
10 taken to puncture the residual blastocoel of neurulae and tadpoles as well as the archenteron. Embryos were rewashed at room temperature in 100% ethanol for two hours to remove residual lipid. After hybridization, staining was allowed to develop overnight and the embryos were then fixed in Bouin's. Newly stained embryos have  
15 a high background of pink stain but most of this washes out, leaving the specific stain. Following overnight fixation, the embryos were washed well with 70% ethanol, 70% ethanol buffered with PBS and methanol. Embryos were cleared in Murray's mix and photographed with Kodak Ektar 25 film, using a Zeiss axioplan microscope (2.5 or  
20 5x objective with 3x12B telescope to assist with focusing).

#### Lineage Tracing

- Lineage tracing with mRNA that encodes nuclear localized B-galactosidase was as we described in S&H, supra. Ventralized  
25 embryos were coinjected at the 32 cell stage with 0.5 ng B-galactosidase and 25 pg noggin $\Delta 5'$  mRNAs. Embryos were fixed and stained with X-gal at approximately stage 22.

## Results

### Noggin cDNA Encodes a Novel Polypeptide

The 1833 nucleotide sequence of the selected clone is shown by SEQ ID NO: 8 and sometimes also referred to as "clone A3." The sequence contains a single long open reading frame encoding a 222 amino acid polypeptide with a predicted molecular weight of 26 kDa. At the amino terminus, the hydrophobic stretch of amino acids suggests that the polypeptide enters the secretory pathway. There is a single potential site for N-linked glycosylation at amino acid 61. Extensive untranslated regions are located both 5' and 3' to the reading frame (593 and 573 bp, respectively). The 3' untranslated region is particularly rich in repeated dA and dT nucleotides, and contains, in addition to a polyadenylation signal sequence located 24 bp upstream from the start of the poly A tail, a second potential polyadenylation sequence 147 bp further upstream.

Sense RNA synthesized from clone A3 with SP6 RNA polymerase was translated in a rabbit reticulocyte lysate system. The 3S-labeled products were fractionated on a 12% SDS-polyacrylamide gel and visualized by fluorography. The major protein product had the expected molecular weight of approximately 26 kDa.

Comparison of the amino acid sequence of the predicted polypeptide to the National Center for Biotechnology Information BLAST network (non-redundant data base) did not identify any similar sequence. Thus, this clone encodes the new type of protein we have named "noggin" which is secreted, and which has dorsal inducing activity in *Xenopus*.

Noggin mRNA can Rescue a Complete Dorsal-Ventral Axis

Injection of noggin RNA into a single blastomere of a four cell stage UV-ventralized embryo can restore the complete spectrum of dorsal structures. The degree of axis rescue was dependent upon the amount of RNA injected, with embryos receiving low doses having only posterior dorsal structures, while embryos receiving higher doses had excess dorsal-anterior tissue. RNA transcripts from two noggin plasmids were tested. The first contained the full cDNA. The second (pNoggin $\Delta$ 5') had a deletion removing the first 513 nucleotides of the 5' untranslated region up to the EcoRI site. The resulting embryos from injection of RNA transcripts of these two plasmids, as well as Xwnt-8 RNA for comparison, were scored according to the dorsoanterior index (DAI) scale of Rao and Elinson (Dev. Biol., 127, 64-77, 1988). In this scale, a completely ventralized embryo is scored as zero, a normal embryo is scored as 5, and the most severely dorsoanteriorized embryos, those having radial dorsoanterior structures, were scored as 10. RNA synthesized from pNoggin $\Delta$ 5' (noggin $\Delta$ 5' mRNA) repeatedly gave a higher DAI than the equivalent amount of mRNA synthesized from the complete cDNA. The dose-dependency of axis rescue by noggin $\Delta$ 5' mRNA was very similar to that of Xwnt-8 mRNA.

UV treated embryos were also injected with a higher doses (1,000 pg) of the noggin mRNAs. Injection of this dose of noggin mRNA into one blastomere at the four cell stage resulted in embryos with very severe hyperdorsalization (DAI >7). However, most of these embryos died at the late gastrula/early neurula stage. Apparently excessively strong gastrulation movements resulted in

the thinning and rupture of the blastocoel roof. We have also observed this effect with high doses of injected Xwnt-8 mRNA.

The rescue of dorsal development by both *noggin*Δ5' and Xwnt-8 mRNAs followed a consistent pattern in which increasing amounts of the mRNAs lead to progressively more anterior structures being rescued. For example, embryos that received 1 pg of the RNAs had primarily the posterior and trunk dorsal structures rescued, and for the most part lacked head structures. Higher doses (10 or 100 pg) of both of the RNAs resulted in embryos with more anterior development, and many had either nearly normal or hyperdorsalized phenotypes.

#### Noggin Injected Blastomeres Act as a Nieuwkoop Center

The effect of varying the site of *noggin* mRNA injection was investigated. Thirty-two cell stage UV-treated embryos were injected with either 0.5 ng of B-galactosidase mRNA alone or 0.5 ng B-galactosidase mixed with 25 pg *noggin*Δ5' mRNA. Injection of *noggin* mRNA into blastomeres of the vegetal tier gave the most strongly dorsoanteriorized embryos. In both of the vegetal injected embryos the nuclear X-Gal staining was found almost exclusively in the endoderm (the mRNA encodes a B-galactosidase that translocates to the nucleus, allowing distinction from the diffuse background stain). One of the embryos shown was strongly hyperdorsalized (DAI approximately 7) as a result of the *noggin* mRNA injection, and had a severely truncated tail and enlarged head structures. Embryos were also rescued by *noggin* mRNA injections into the marginal zone. In these embryos B-galactosidase staining was observed primarily



in the axial and head mesoderm. Injection of noggin mRNA into the animal pole had very little effect on axis formation. Likewise, B-galactosidase mRNA alone was without effect.

5 Noggin mRNA is Expressed Both Maternally and Zygotically

In northern blot analysis of RNA from *Xenopus* embryos two noggin mRNA species of approximate sizes 1.8 and 1.4 kb were observed. A relatively low level of noggin mRNA was detected in oocytes. By stage 11 the level of noggin mRNA was significantly  
10 higher, reflecting zygotic transcription (as opposed to the maternally deposited transcripts seen in oocytes). Noggin mRNA remained at the elevated level up to the latest stage examined (stage 45).

We expect that the primary dorsalizing RNA in our library to  
15 be elevated in LiCl-treated embryos relative to normal or UV-treated embryos. Lithium ion treatment resulted in a large increase in the amount of noggin mRNA expressed, relative to untreated embryos.

UV treatment had the opposite effect. Noggin mRNA expression was  
20 essentially undetectable in total RNA samples from these embryos. Thus, the abundance of noggin mRNA in manipulated embryos parallels the rescuing activity.

We analyzed the distribution of noggin in oocytes and cleavage stage embryos. Since the amount of maternally deposited noggin  
25 RNA is too low for in situ hybridization to detect above background, we used an RNase protection assay. Oocytes were dissected into animal and vegetal halves. No enrichment of noggin mRNA was seen in either hemisphere relative to total oocyte RNA. Four-cell stage

embryos were dissected into dorsal and ventral halves, as well as animal and vegetal halves. Noggin transcripts were found to be distributed evenly between dorsal and ventral hemispheres as well as animal and vegetal hemispheres. The same result was obtained with embryos that were tilted 90° immediately following fertilization and then marked with a vital dye on their uppermost side to indicate the future dorsal side. Older (32 cell stage) blastula embryos were also dissected into dorsal-ventral and animal-vegetal halves. No enrichment of noggin mRNA in any of the hemispheres was seen relative to the total embryo. In addition, treatment did not alter the abundance of maternally deposited noggin RNA, indicating no preferential degradation in ventral tissues. Samples with known amounts of in vitro synthesized noggin mRNA were included in the RNAase protection assay. From these and other data we estimate that there is approximately 0.1 pg of noggin mRNA per blastula stage embryo and 1 pg per gastrula stage embryo.

The localization of noggin transcripts was investigated in early gastrula stage embryos. Dorsal lips were dissected from stage 10.5 embryos. A northern blot of equal amounts of total RNA from intact embryos, dissected dorsal lips, and from the remaining embryo after dissection of the dorsal lip was hybridized with a noggin probe and then re-hybridized with an EF1a probe, as a control for amount of RNA loaded per sample. The autoradiograph of the blot showed that noggin mRNA at this stage is enriched in the dorsal lip.

In situ Hybridization: Zygotic Expression of Noggin in the Spemann Organizer

The localization of noggin transcripts in developing embryos was examined in greater detail using whole mount in situ

hybridization. Whole fixed embryos were hybridized with digoxigenin containing RNA probes.

Hybridized RNA probe was then visualized with an alkaline phosphatase-conjugated anti-digoxigenin antibody. The specificity of hybridization seen with antisense noggin probes was tested both by hybridizing embryos with sense noggin probes, and by using two non-overlapping antisense probes. Due both to the low level of expression, and to background staining, noggin mRNA could not be detected unequivocally before the late blastula stage. The increased level of noggin mRNA that was detected by northern blot following activation of zygotic transcription was apparent in *in situ* hybridization at stage 9 as a patch of staining cells on the dorsal side of the embryo. Viewed from the vegetal pole, this patch of cells was restricted to a sector of about 60°. A side view of the same embryo shows that the staining cells were located within the marginal zone (i.e., between the animal and vegetal poles and within the presumptive dorsal mesoderm forming region). Transcripts are largely restricted to the nucleus at this stage.

A side view of an early gastrula stage embryo (approximate stage 10.5) shows specific hybridization primarily in the involuting mesoderm at the dorsal lip. A vegetal view of the same embryo (blastopore lip arrowed) shows that noggin mRNA is most abundant on the dorsal side, but expression extends at the lower level to the ventral side of the embryo. This method of *in situ* hybridization does not detect transcripts in the most yolky endodermal region of embryos, therefore we cannot rule out expression in more vegetal regions than those seen in the Figure. Treatments which are known to affect the size of the dorsal lip

(LiCl treatment, UV irradiation) had a profound effect on the pattern of *noggin in situ* hybridization. In LiCl treated embryos the staining is intense throughout the marginal zone. UV treatment reduced the hybridization signal to low levels. This result is  
5 consistent with amounts of *noggin* mRNA seen by northern blot analysis. The UV treated embryo also is a negative control for specificity of hybridization.

As gastrulation proceeds, *noggin* mRNA staining follows the involuting dorsal mesoderm, and is highest in the presumptive  
10 notochord. By the late neurula stage (approximately 18) *noggin* mRNA expressing cells are clearest in the most dorsal mesoderm, primarily in the notochord but also extending more anteriorly into the pre-chordal mesoderm. The anterior tip of the notochord is arrowed. During tailbud stages expression of *noggin* in the dorsal  
15 mesoderm declines, though expression in the notochord persists in the growing tailbud. Expression of *noggin* initiates at several new sites, which become progressively clearer as the tadpole matures. A discontinuous line of stained cells runs the length of the roof plate of the neural tube. Staining is also apparent in the head mesoderm,  
20 primarily in the mandibular and gill arches. We suspect that this expression corresponds to skeletogenic neural crest cells. Furthermore, subsets of these cells express homeobox genes that mark different anterior-posterior levels of the head neural crest, for example *En-2* in the mandibular arch is seen by antibody  
25 staining. Cells with stellate morphology stained from *noggin* mRNA in the tail fin. These stellate cells are also likely to be derived

from the neural crest. None of these patterns were seen with the sense probe, or with a number of other probes.

## EXAMPLE 2

5

### Noggin cDNA Transfected into COS Cells Produces Active Conditioned Medium

For COS cells the noggin cDNA was inserted into a COS cell  
 10 expression vector. COS cells were transfected, and medium  
 harvested after allowing expression of the introduced noggin genes.  
 This medium has been tested in an animal cap assay for mesoderm  
 inducing or dorsalizing activity. We have tested two transfection  
 protocols, a standard one, where cells recover and then are  
 15 transferred to serum-free medium, and an alternate where cells are  
 transferred to a defined medium lacking serum but containing  
 transferrin, insulin, and BSA. Cells remain healthy in the  
 supplemented medium and a cotransfected  $\beta$ -galactosidase gene  
 gives 100 fold more activity than in the unsupplemented medium.  
 20 The results of treating cells with these media is shown below in  
 Table 1. Animal caps were taken from ventralized animals, treated  
 and at the end of neurulation they were scored for elongation,  
 usually a sign that notochord or neural tissues have been induced.  
 Elongation is indicated in Table 1 by a "+" and even greater  
 25 elongation a "++." In addition, they are scored for a molecular  
 marker by Northern blotting.

As shown by the data of Table 1, the noggin cDNA has a large  
 effect on the COS cell conditioned medium. However, noggin is  
 probably interacting with something else in the medium, since COS-

cell conditioned medium alone has some activity. It is possible that noggin is causing the cells to secrete something that they normally would not, but the experiments do indicate that noggin is secreted and is responsible for some of the activity.

TABLE 1

Cos Cell Conditioned Medium: Effects on Animal Caps

		Elongation	N-CAM
	expression		
5	Transferred to serum free medium + transferrin, BSA, and insulin		
10	1. Vector only	+/-	+
	2. Noggin cDNA	++	++
	Transferred to serum free medium without supplements		
15	1. Vector only	-	-
	2. Noggin cDNA	-	-
20	<u>Noggin mRNA Injected into Oocytes Produces Active Secreted Noggin Protein</u>		

A second approach to studying whether protein can be secreted in active form is to inject oocytes with mRNA and take material

secreted by the oocyte. A particular advantage of this method is that the injected mRNA is efficiently translated, and most of the translation of the oocyte can be taken up by the injected mRNA. A new protein, whose synthesis is directed by injected noggin mRNA is secreted into the medium. Noggin clearly synergizes with activin

to produce elongated explants that express elevated levels of muscle actin.

Biochemical Properties of Noggin

Injected oocytes are injected with mRNA, and labelled with <sup>35</sup>S methionine. Most of the radioactive protein secreted into the medium is from the injected mRNA. The noggin protein, which is almost isotopically pure, can then be analyzed. From this analysis we have determined that noggin is a dimeric glycoprotein. When run under reducing conditions, and treated with N-glycanase to remove sugar residues, noggin migrates only slightly slower than its predicted molecular weight of 26 kDa. The removal of sugar side chains results in a loss of about 4 kDa from a starting apparent molecular weight of 33 kDa. When run under non-reducing conditions it migrates at double this value.

We do not yet know if the dimer of the protein is the active species, or if there is a proteolytically processed form which is active. In a control experiment with activin mRNA, oocytes produce activin activity, but the bulk of the radiolabelled protein migrates as the precursor form. Only a small amount of processed protein (15 kDa) was detected. It is possible that noggin injected oocytes secrete predominantly unprocessed protein and a trace of extremely active processed protein that we have not detected. Despite the caveats, the main point from analysis of injected oocytes and transfected COS cells is that active noggin can be obtained as a freely soluble secreted polypeptide. This sets it apart from the other group of genes with dorsalizing activity, the wnts. Wnt proteins have not been available in soluble form and this has greatly hampered the analysis of their biological activities, and of the receptor that binds to them.



EXAMPLE 3Cloning of the Mouse Noggin Homolog

It is currently impossible to eliminate zygotic noggin transcription from developing *Xenopus* embryos. In contrast, it should be possible to generate homozygous null mutations in the mouse. We have cloned the mouse noggin cDNA (SEQ ID NO: 10). This is useful to generate mutant mice. In addition to generating the probes and tools to make mutant mice, a comparison of the noggin sequences should be a useful predictor of conserved domains and functions. The C-terminal 80 amino acids are 87% identical between SEQ ID NOS: 8 and 10.

Mouse noggin was isolated from an embryonic cDNA library by probing with a radiolabelled frog noggin cDNA under conditions of moderate stringency (as defined earlier). Subsequently a genomic clone was isolated by probing a genomic library with the mouse noggin cDNA under conditions of high stringency (as defined, but hybridized at 42° C and washed at 50°C in 15 mM NaCl, 1.5 mM sodium citrate). The full nucleotide sequence of mouse noggin cDNA (SEQ ID NO: 25) as well as the deduced amino acid sequence (SEQ ID NO: 26) are shown in Figure 13. There are only two amino acid differences between mouse noggin and human noggin.

25

EXAMPLE 4Cloning of the Human Noggin Homolog5 Materials and MethodsProbe preparation

Two oligonucleotides were synthesized based on the mouse noggin sequence (supra). The sequence of the oligonucleotides is noggin 5' : 5'-CAG ATG TGG CTG TGG TCA-3' (SEQ ID NO: 18)

- 10 corresponding to amino acids QMWLWS (SEQ ID NO: 19) and noggin 3' : 5'-GCAGGAACACTTACACTC-3' (SEQ ID NO: 20) corresponding to amino acids ECKCSC (SEQ ID NO: 21) of the mouse noggin protein .

- The oligonucleotides were used for PCR amplification of a segment of DNA of 260 nucleotides using as a template a mouse
- 15 cDNA clone prepared as set forth in Example 3. The amplified fragment had a nucleotide sequence that corresponds to nucleotides 2 through 262 of the mouse sequence as set forth in SEQ ID NO: 10. After amplification, the PCR reaction was electrophosed in agarose gels, the DNA band of 260 nts purified by Magic PCR (Promega), and
- 20 used as template for the probe labeling reaction. The probe was labeled using a standard PCR reaction (Perkin-Elmer) on 20 ng of DNA template and 0.2 m Curie of alpha 32P-dCTP (Du Pont 3000 Ci/mmol) instead of dCTP. Unincorporated label was separated from the probes on a G50 NICK column (Pharmacia). The excluded volume
- 25 of the reaction contained a total of 1.8 x10<sup>8</sup> cpm.

In addition, one degenerated oligonucleotide, named noggin D, corresponding to conserved mouse and Xenopus noggin sequences, was synthesized as follows: Noggin D: 5'- GARGGIATGGTITGYAARCC-

3' (SEQ ID NO: 22). Noggin D (SEQ ID NO: 22) was labeled by kinase reaction using T4 polynucleotide kinase and gamma 32P ATP. The labeled oligonucleotide was purified by NAP5 (Pharmacia) column and used for library hybridization.

5

#### Library screening

A human placental genomic library (Clontech Cat#HL1067J, average insert size 15 kb) in vector EMBL-3 was plated according to manufacturer specifications in NM 538 E.coli. Approximately 3 million plaques were transferred to nitrocellulose filters (BA-85 Schleicher and Schuell) in three replicas (named A, B and C) and screened according to Maniatis, et al.[Sambrook, et al., Molecular cloning a laboratory manual, CSH Lab Press, New York (1989)]. The replica filters A and C were hybridized in a buffer containing 0.5 M sodium phosphate, pH 7.2, 7% sodium dodecyl sulphate, 1% crystalline BSA, 1 mM EDTA, 40 mg/ml denaturated salmon sperm DNA and about 1x10<sup>6</sup> cpm/ml of the PCR probe (supra). After hybridization for 12 h at 65°C, the filters were washed twice at room temperature in 2x SSC (30 mM sodium citrate, 0.3 M NaCl), 0.1% SDS and then at 65°C in 2xSSC, 0.1% SDS for 20 min and exposed to Kodak X-OMAT AR film. The filter replica B were hybridized with the labeled oligonucleotide noggin D in 6xSSC, 0.1% SDS at 51°C for 12 h followed by wash at 2xSSC, 0.1% SDS at room temperature, and in 6xSSC, 0.1% SDS at 50°C and exposed to Kodak X-OMAT AR film. Positive plaques from all replicas were isolated and purified by re-screening as above. Purified positive plaques were suspended in 500 µl SM (100 mM NaCl, 10mM MgSO<sub>4</sub> x 7H<sub>2</sub>O, 50 mM Tris HCl pH 7.5, 0.01% gelatin). 160 µl of phage suspension was

mixed with 0.5 ml saturated NM538 culture, incubated for 20 min at 37°C and then inoculated into 250 ml LB containing 10 mM Mg SO<sub>4</sub>, 0.2% maltose. The cultures were incubated until cell lysis (7-8 hr) at 37°C. The phage lysates were used for phage DNA purification by the Qiagen procedure according to the manufacturers recommendations (Qiagen).

### Sequencing

Sequencing was performed by using the Applied Biosystems Model 373A automatic sequencer and Applied Biosystems Taq DyeDeoxy™ Terminator Cycle Sequencing Kit.

### Results

Filters hybridized to the PCR mouse noggin probes (SEQ ID NOS: 18 and 20) showed two strong signals corresponding to phage plaques named hnogλ-9 and hnogλ-10. These plaques also hybridized to degenerate oligonucleotide probe nogginD (SEQ ID NO: 22) revealed that these clones correspond to the human noggin gene. In addition, two other plaques named hnogλ-5 and hnogλ-7 produced slightly weaker signals when hybridized to the PCR probes. These clones correspond to either human noggin or related gene(s). All of the human DNA inserts can be excised from the vectors using known restriction sites as described in the literature regarding each particular library.

A 1.6 kb SacI fragment from clone hnogλ-9 containing the human noggin gene was subcloned and the nucleotide sequence determined as set forth in Figure 1. The amino acid sequence for human noggin, as deduced from the nucleotide sequence, is also set

forth in Figure 1. The gene or cDNA may be expressed in various eukaryotic or prokaryotic expression systems to produce biologically active human noggin protein. It is expected that the human protein will exhibit neurotrophic activity similar to that exhibited by *Xenopus* noggin protein.

### EXAMPLE 5

#### Tissue Localization of message for human noggin

##### 10 Materials and Methods

##### Probe preparation

Probes were prepared as set forth in Example 4. The oligos used are as follows:

15 SEQ ID NO: 23:

5' GAC.TCG.AGT.CGA.CAT.CGC.AGA.TGT.GGC.TGT.GGT.CAC

SEQ ID NO: 24:

5' CCA.AGC.TTC.TAG.AAT.TCG.CAG.GAA.CAC.TTA.CAC.TCG.G

(The underlined sequence represent mouse noggin sequence; the rest of the sequence are tails containing restriction sites for cloning.)

A DNA fragment of approximately 300 bp was obtained by PCR amplification of a mouse cDNA clone prepared as described in Example 3.

### RNA Preparation and Northern Blots

Selected tissues were dissected from Sprague-Dawley rats and immediately frozen in liquid nitrogen. RNAs were isolated by homogenization of tissues in 3 M LiCl, 6 M urea, as described in

5 Bothwell, et al. 1990 (Methods of Cloning and Analysis of Eukaryotic Genes, Boston, MS, Jones and Bartlett). RNAs (10 µg) were fractionated by electrophoresis through quadruplicate 1% agarose-formaldehyde gels (Bothwell, et al., 1990, Methods of Cloning and Analysis of Eukaryotic Genes, Boston, MS, Jones and Bartlett)

10 followed by capillary transfer to nylon membranes (MagnaGraph, Micron Separations Inc.) with 10xSSC (pH7). RNAs were UV-cross-linked to the membranes by exposure to ultraviolet light (Stratalinker, Stratagen, Inc.) and hybridized at 68°C with radiolabeled probes in the presence of 0.5 M NaPO<sub>4</sub> (pH 7), 1% bovine

15 serum albumin (fraction V, Sigma, Inc.) 7% SDS, 1 mM EDTA [Mahoudi, et al., Biotechniques 7:331-333 (1989)], 100 µg/ml sonicated, denatured salmon sperm DNA. Filters were washed at 68°C with 3xSSC, 0.1% SDS and subjected to autoradiography for 1 day to 2 weeks with one or two intensifying screens (Cronex, DuPont) and X-

20 ray film (AR-5, Kodak) at 70°C. Ethidium bromide staining of the gels demonstrated that equivalent levels of total RNA were being assayed for the different samples [as in Maisonpierre, et al., Science 247:1446-1451 (1990)].

RNA was prepared from the following human cell lines:

	<u>Neuroblastoma</u>	<u>Neuroepithelioma</u>	
5	CHP-134	SK-N-MC	
	LA-N-1	CHP-100	
	LA-N-5	IARC-EWI	
	IMR-32	SK-N-LO	
	SHSY5Y	SK-ES	
10	SKNSH	DADY	
	SHEP		
	<u>Hematopoietic</u>	<u>Small Cell Lung Carcinoma</u>	<u>Cervical Carcinoma</u>
15	K562	Calu 3	HeLa
	U937	SKLu	
	M1	NCI-H69	
	TF1	SKMES	
	BAF		
20	B9		

Sympathoadrenal PrecursorHepatoblastomaMedulloblastoma

MAH

HEPG2

Madsen

5

Med

U266

Pheochromocytoma

PC12

10 RESULTS

We have amplified a DNA fragment from the mouse noggin plasmid, corresponding to the region conserved between *Xenopus* and mouse noggin.

15 The amplified fragment of approximately 300bp was used as probe to hybridize to northern blots, with RNAs prepared from adult and embryonic tissues, as well as from various cell lines. Noggin transcript of about 2kb in size was detected in adult rat brain, and in a cell line, SKMES, a small cell lung carcinoma.

20 Expression of noggin transcripts was examined in various tissues from rat and mouse at different stages of development and in adult. In the mouse, noggin transcripts can be detected in embryos or head from E9 to E12, as well as in newborn brain and adult brain. There was no detectable signal in peripheral tissues  
 25 examined except in skeletal muscle. Abundant level of expression was also found in hippocampal astrocytes isolated from postnatal mouse.



In the rat, noggin transcripts were detectable in embryos or head from E9 to E18, as well as in brain from P1, P19 and adult brain. In the cerebellum, expression of noggin appeared to be higher in E18 and P1; in the spinal cord, expression of noggin mRNA peaked at P1. Examination of noggin expression in all of the CNS regions, especially the olfactory bulb, midbrain, hindbrain and cerebellum. In the adult, noggin mRNA could be detected in all CNS regions, especially the olfactory bulb and cerebellum. There also appeared to be low levels in the skin.

### EXAMPLE 6

#### 15 Neural Induction by Noggin

##### Materials and Methods

##### Preparation of Xenopus noggin CHO cell conditioned medium

Xenopus noggin CHO conditioned medium was made by selecting for stably transfected CHO cells. Dihydrofolate reductase (DHFR) deficient CHO parental cells ( J. Papkoff, Syntex Research) were transfected with a Xenopus noggin expression plasmid containing noggin in tandem with the dihydrofolate reductase gene. Growth in nucleoside free medium was used to select for successfully transfected cells. Nine colonies of transfectants were picked and grown up individually. The noggin gene in these cells was amplified by slowly increasing the dose of methotrexate, an inhibitor of DHFR. The presence of noggin transcripts was first tested by Northern

analysis. Subsequently, two clones, B3 and C3, were shown to secrete noggin protein, since conditioned medium from these lines was capable of dorsalizing ventral marginal zones. Furthermore, by labeling B3 cellular proteins with 35S-methionine, noggin protein could be identified as a band of about 30kD on reducing SDS-PAGE, and a band of 60kD on non-reducing SDS-PAGE indicating it forms the expected dimer. These properties matched those of the noggin protein previously produced in *Xenopus* oocytes supra, (Smith et al., Nature 361, 547-49, 1993). B3 conditioned medium was collected in a mixture of 1 part alpha MEM and 9 parts CHO-S-SFMII (Gibco-BRL). The cells were allowed to condition the medium for 3 days. Control medium from parental cells (CHO dhfr-) was collected identically. Twenty fold concentrated medium was made using Centriprep 10 concentrators, where the 20 fold change is measured by volume.

15

#### Purification of human noggin from COS cells

Human noggin protein was purified by a cationic exchange column. COS/M5 cells were transiently transfected with a human noggin expression plasmid, pCAE11. Cells were allowed to condition DMEM (Specialty Media) for two to three days, after which the medium was removed. Particulates from the medium were removed by a centrifugation step and subsequent passage through a 0.2um cellulose acetate filter. This cleared medium was pumped onto a MonoS (Pharmacia) column which was washed with several volumes 40mM sodium phosphate (pH 7.3), 150mM NaCl, 1mM EDTA. Proteins were then eluted in a linear gradient with 40mM sodium phosphate

(pH 8.5), 1.8M NaCl, 1mM EDTA. Noggin protein elutes at 0.8M NaCl and is  $\geq 90\%$  pure by SDS-PAGE.

#### Xenopus otx isolation

- 5 To isolate Xenopus Otx clones a tadpole head cDNA library (Hemmati-Brivanlou, et al., Development 106, 611-617, 1989) was screened with a mouse otx cDNA (S-L Ang and Rossant, Toronto) at low stringency. The clones that were picked fell into two classes. One class, which we have designated otxA, included
- 10 pXOT21.2, the probe used here. By in situ hybridization, transcripts are first detected prior to gastrulation in the superficial layer on the dorsal side. During neurulation a large anterior domain expressed the gene, and includes both neural and non-neural tissues. After a decline in expression in the tailbud tadpole, the gene is
- 15 reexpressed specifically in the brain and eyes.

#### Ventral marginal zone assay

##### Embryo preparation

- Xenopus laevis embryos are fertilized and de-jellied as
- 20 described (Condie and Harland, 1987. Development 101, 93-105), routinely the evening before dissections. Embryos are cultured overnight at 15°C. The vitelline membrane surrounding each developing embryo is manually removed the following morning at the late blastula stage. Until dissection, the embryos are maintained in
- 25 1/3x modified ringers in agarose coated dishes.

### Ventral marginal zone dissection

Embryos are oriented with their yolky vegetal hemisphere up so the dorsal side can be identified. The dorsal side of the early gastrula is marked by the presence of a small arc of dense pigment called the "dorsal lip" which marks the start of involution of dorsal mesoderm. The ventral marginal zone (VMZ) is found directly opposite the dorsal lip, and is dissected. Since the vitelline membrane has been removed, the embryo tends to flatten. Using a specially constructed knife made of an eyebrow, mounted onto a glass pipet with wax, two cuts are made through the flattened embryo from the top facing vegetal pole through to the animal pole. The cuts are made such that they isolate approximately 30-60 degrees of the ventral side away from more lateral tissues. A third cut which is perpendicular to the first two cuts completely isolates the ventral marginal zone tissue away from the rest of the embryo. This third cut is at the level of approximately two thirds of the radius of the embryo from the center. Prior to treatment the VMZ is washed 1x in the culture medium.

### 20 Assay

Approximately between 5 to 10 VMZs are used per assay. The washed VMZs are dropped gently (trying to minimize transfer of liquid) into eppendorf tubes containing the desired treatment protein medium for assay. The VMZs are allowed to develop to the late neurula or early tailbud stage as assessed by control whole embryo development. At this time RNA is isolated from the VMZs and control whole embryos as described (Condie and Harland, *ibid*). The expression of muscle actin in VMZs indicates a dorsalization event

(Lettice and Slack, 1933. Development, 117, 263-72) . RNA from each sample is run on a formaldehyde-agarose gel and blotted to gene screen. The blot is then hybridized with a Xenopus muscle actin probe (Dworkin-Rastl et al., 1986. J. Embryol. exp. Morph. 91, 153-68) . Quantitation of dorsalization can be carried out by normalizing muscle actin signal to that of the ubiquitously expressed EF-1 $\alpha$  (Krieg et al., 1989. Devl. Biol. 133, 93-100) . Quantitation is done using phosphor imaging.

#### 10 RNase protection assay

RNase protection was carried out as described (D.A. Melton et al., Nucleic Acids Res 12,7035-56, 1984), with the modification that digestion was carried out at room temperature (22°C) using RNase T1 only (Calbiochem 556785) at 10 units/ml. 20-30 animal caps were harvested for each lane, of this 80% was used for neural markers and 10% for muscle actin and collagen type II. For goosecoid and brachyury 20 caps were used. Exposures ranged from 12 hours to 5 days. In all cases, films were preflashed. In cases where a marker was not expressed, the result was confirmed with greater sensitivity using phosphor imaging.

#### Results

The development of vertebrate embryos requires several inductive interactions. Mesoderm, which eventually forms tissues such as notochord, muscle, heart, mesenchyme and blood, is induced in the equatorial region of the embryo (Nieuwkoop, Wilhelm Roux' Arch. EntwMech. Org, 162, 341-373, 1969) . This inductive event is well studied, and there are several candidates for the endogenous

- inducer(s) including members of the fibroblast growth factor(FGF) family and activin (Jessell and Melton, Cell 68, 257-70 1992; Sive, Genes Dev 7, 1-12, 1993) and TGFb family (Asashima, et al., Roux's Arch. Dev. Biol. 198, 330-335, 1990; Asashima, et al.,
- 5 Naturwissenschaften 77, 8, 389-91, 1990; Green and Smith, Nature 347, 391-394, 1990; Smith, et al., Nature 345, 6277, 729-31, 1990; Thomsen, et al., Cell 63, 485-493, 1990; van, et al., Nature 345, 6277, 732-4, 1990) . The use of dominant negative receptors for both FGF (Amaya, et al., Cell 66, 257-270, 1991) and activin
- 10 (Hemmati-Brivanlou and Melton, Nature 359, 609-614, 1992) in Xenopus embryos strongly suggests that the signaling pathways activated by these molecules are essential for proper mesoderm formation . Molecules such as wnts (Christian, et al., Development 111, 1045-1055, 1991; McMahon and Moon, Cell 58, 1075-84, 1989;
- 15 Smith and Harland, Cell 67, 753-765, 1991; Sokol, et al., Cell 67, 741-752, 1991) and noggin (Smith, et al., Nature 361, 547-49, 1993) modify the kinds of mesoderm made without inducing mesoderm directly.

- In a subsequent induction, the dorsal mesoderm of the Spemann
- 20 organizer signals nearby lateral mesoderm to take on a more dorsal fate (Dale and Slack, Development 100, 2, 279-95, 1987; Lettice and Slack, Development, 117, 263-271, 1993; Spemann and Mangold, Arch. Mikrosk. Anat. EntwMech. 100, 599-638, 1924; Stewart and Gerhart, Development 109, 363-372, 1990) . The only known
- 25 factor which is expressed in the organizer and can mimic its dorsalizing activity is noggin.

Dorsal mesoderm of the Spemann organizer also signals nearby ectoderm to become neural tissue. Neural induction by dorsal

mesoderm has been demonstrated in amphibians (Dixon and Kintner, Development 106, 749-757, 1989; Doniach, et al., Science 257, 5069, 542-5, 1992; Hamburger, The Heritage of Experimental Embryology: Hans Spemann and the Organizer, 1988; Kintner and Melton, Development 99, 311-25, 1987; Spemann, Arch. mikrosk. Anat. EntwMech. 100, 599-638, 1938) , birds (Kintner and Dodd, Development 113, 1495-1506, 1991; Tsung, et al., Acta Biol exp Sinica 10, 69-80, 1965) , and recently in mice (Ang and Rossant, Development 118, 139-149, 1993) . Despite decades of effort, little is known about the molecular nature of the factors responsible for this induction. Among known inducers, activin can promote formation of neural tissue, but this is due to a secondary induction by the dorsal mesoderm that activin induces (Green, et al., Development 108, 1, 173-83, 1990; Green and Smith, Nature 347, 391-394, 1990; Kintner and Dodd, Development 113, 1495-1506, 1991) . Thus, activin cannot promote formation of neural tissue when added to gastrula ectoderm; however, such ectoderm remains competent to be neuralized by dorsal mesoderm until the end of gastrulation (Sharpe and Gurdon, Development 109, 765-74, 1990) .

#### Direct Neural Induction by Noggin

Candidates for the endogenous inducer are expected to induce neural tissue in the absence of dorsal mesoderm. Competent animal cap ectoderm from late blastula stage embryos (St9) was used to test noggin's neural inducing capacity. Xenopus noggin protein conditioned medium was collected from stably transfected CHO cells and twenty fold concentrated medium was used to treat St 9 animal

caps. Markers used in an RNase protection assay were N-CAM (Jacobson and Rutishauser, *Developmental Biology* 116, 524-31, 1986; Kintner and Melton, *Development* 99, 311-25, 1987), a neural cell adhesion molecule, a neural specific isoform of b-tubulin (Good, et al. *Nucleic Acids Res* 17, 8000, 1989; Good, et al., *Dev Biol* 137, 414-8, 1990; Richter, et al., *Proc Natl Acad Sci USA* 85, 8086-90, 1988) that is expressed in the hind brain and spinal cord, and XIF3, a neurally expressed intermediate filament gene (Sharpe, et al., *Development* 107, 701-14, 1989) to assay for neural induction. All these markers are restricted to neural tissue, however, only NCAM is expressed throughout the nervous system. We found that *Xenopus*-noggin conditioned medium induces high levels of N-CAM and XIF3 expression [Fig. 2.; lane8] in treated animal caps, without inducing muscle actin (lane 13) (Dworkin-Rastl, et al., *J. Embryol. exp. Morph.* 91, 153-168, 1986; Mohun, et al., *Nature* 311, 716-721, 1984). Control CHO cell medium induces neither muscle nor neural tissues (lanes 7,12). St 9 activin treated animal caps express muscle actin (lane11) and all three neural markers (lane 6), demonstrating activin's ability to generate neural tissue indirectly. It is interesting to note that noggin induces very little, if any b-tubulin expression, while inducing high levels of N-CAM, but activin induction has nearly the converse effect.

To determine whether noggin protein is sufficient to induce neural tissue, COS cells were transfected with pCAE11, a human noggin expression plasmid, and the conditioned medium was purified by cation exchange chromatography resulting in noggin preparations that were 90% pure [Fig. 3.]. Such purified human noggin protein is also able to induce neural tissue in animal caps [Fig. 4a., see below].



We have shown that noggin does not induce muscle in late blastula stage animal caps, however, it is possible that noggin induces other types of dorsal mesoderm. To address this concern, we asked whether noggin could induce the expression of the early mesoderm markers goosecoid (Blumberg, et al., Science 253, 194-6, 1991; Cho, et al., Cell 67, 1111-20, 1991), a marker of organizer tissue and subsequently head mesoderm or X-brachyury (Smith, et al., Cell 67, 79-87, 1991), which appears to be expressed in all mesodermal precursors early, and subsequently is expressed in posterior mesoderm and notochord. Animal caps were treated at stage 9 and collected at stage 11, when expression of goosecoid and brachyury in the normal embryo is high. Neither marker is turned on by purified human noggin (Fig. 4b. lane 5) at a dose with demonstrated neural inducing activity (Fig 6 lane15); in contrast animal caps treated in the same fashion with activin show both goosecoid and X-bra expression (Fig. 4b. lane 4) as expected for this mesoderm inducing factor (Cho, et al., Cell 67, 1111-20, 1991; Smith, et al., Cell 67, 79-87, 1991). Untreated animal caps show no expression of these mesodermal markers (lane 3), and RNA levels in the collected animal caps are shown to be comparable using EF-1a levels (Krieg, et al., Dev Biol 133, 93-100, 1989).

Since purified human noggin is capable of driving neural induction, no additional factors which may have been present in the crude conditioned medium are required. Furthermore, *Xenopus* and human noggin, with 80% amino acid identity, can both act to induce neural tissue in *Xenopus*, suggesting a conserved function for these two proteins. However, for noggin to be a candidate endogenous neural inducer it must be able to induce neural tissue at a stage

when neural induction occurs in normal whole embryos. It is unclear when the first instructive signals are sent from dorsal mesoderm to ectoderm in embryos. However, it is known that by early gastrula stages, dorsal ectoderm has already been specified to become neural tissue (Jones and Woodland, Development 107, 785-91, 1989). The neural inducing signal is therefore likely to start before this stage. The latest stage at which animal caps have been shown to be competent to respond to neural-inducing mesoderm is the early neurula (St13-14) (Sharpe and Gurdon, Development 109, 765-74, 1990). Thus, a candidate endogenous neural inducer must be able to induce neural tissue from gastrula stage competent ectoderm.

#### Neural Induction at the Gastrula Stage

In order to assess the competence of ectoderm to respond to noggin we treated animal caps taken from blastula (St8), late blastula (St9), early gastrula (St10) and ventral animal caps from mid-gastrula (St10.5) stage embryos with purified human noggin[Fig. 2.]. We also treated similarly staged animal caps with activin to demonstrate its mesoderm inducing and secondary neural inducing activities, and to contrast activin's effects with those of noggin[Fig. 4a.]. Activin treated animal caps show neural induction only in conjunction with induction of dorsal mesoderm, such as muscle and notochord (lanes 3,6,9). In a number of experiments, we confirmed that activin's ability to induce dorsal mesoderm, and consequently neural tissue, declines rapidly at the gastrula stage (lane 12) (Green, et al., Development 108, 173-83, 1990; Kintner and Dodd, Development 113, 1495-1506, 1991). In the experiment shown here a larger than usual dose of activin was given. Under

these conditions, only a small amount of neural tissue is made, perhaps because so much mesoderm is induced that there is not much competent ectoderm left in the explant to be neuralized. In contrast noggin can induce neural tissue in animal caps taken from all of  
5 these stages without inducing the notochord and somite marker, collagen type II (Amaya, et al., Development 118, 477-87, 1993; Bieker and Yazdani-Buicky, J Histochem Cytochem 40, 1117-20, 1992), or muscle actin (lanes 4,7,10,13). This gives additional support to the proposal that noggin is a direct neural inducer, since  
10 it can act in the absence of both early and late mesoderm markers. Furthermore, we have shown that noggin can induce neural tissue in competent ectoderm at a time when mesoderm inducers are inactive.

In some experiments, noggin addition to gastrula (but not blastula) animal caps resulted in induction of muscle (data not  
15 shown). This occurred at stages when activin could no longer induce muscle. We interpret this as a result of a dorsalizing action by noggin on tissues that have received a weak mesoderm-inducing signal. The mesoderm-inducing signal which spreads into the gastrula animal cap is not enough to induce mesoderm, but in the  
20 presence of Xwnt-8 or noggin, muscle is formed. One interesting corollary of the induction of muscle is that the kinds of neural tissue seen in the explant are modified. Induction in explants that contain no muscle usually yields N-CAM expression, but if muscle is present, expression of both N-CAM and b-tubulin is seen. This  
25 phenomenon is demonstrated in the secondary neural induction by activin in St. 9 animal caps [Fig. 2.] and in the comparison of neural tissue induced by noggin in ventral marginal zones versus animal caps [Fig. 6.]. In the ventral marginal zones and animal caps in which

muscle is present, both N-CAM and b-tubulin are expressed, whereas induced animal caps without muscle, show only N-CAM expression.

Neural induction after injection of DNA coding for noggin

5 To confirm our conclusions using a different experimental approach, we have directed noggin expression to gastrula stage animal caps by injecting the plasmid pCSKA-noggin into the animal pole of a one cell stage embryo. This plasmid, in which noggin is under the control of the cytoskeletal actin promoter, turns on the  
10 expression of noggin mRNA at the onset of gastrulation (Smith, et al., Nature 361, 547-49, 1993). At the blastula stage, the animal caps are dissected and then matured to tailbud stages for molecular analysis. Animal caps injected with the noggin plasmid show expression of N-CAM in the absence of muscle or notochord markers  
15 (Fig. 4c. lane 2). A control plasmid directing the expression of lac Z showed no neural or mesodermal induction as expected (lane 1). This experiment demonstrates that ectopic noggin expression can directly induce neural tissue in gastrula stage ectoderm, a stage when neural induction is taking place in whole embryos.

20

Differences in competence between dorsal and ventral animal caps.

Animal caps taken from the dorsal side of gastrula stage embryos show greater competence to form neural tissue than ventral animal caps (Otte and Moon, Cell 68, 1021-29, 1992; Sharpe, et al.,  
25 Cell 50, 749-58, 1987), when involuted anterior mesoderm is used as the inducer. This type of mesoderm, however, has weaker inducing capacity than the rest of the involuted mesoderm (Sive, et al., Cell 58, 171-180, 1989). Furthermore, the ventral side of an

embryo can support the formation of a complete secondary axis when the organizer is placed on that side (Gimlich and Cooke, Nature 306, 471-3, 1983; Smith and Slack, J. Embryol. Exp. Morph. 78, 299-317, 1983; Spemann, Arch. Mikrosk. Anat. EntwMech. 100, 599-638, 5 1938), indicating that there is no qualitative difference in competence. Thus, while a weak inducer might unmask slight differences in competence of the ectoderm, it has been suggested that a robust neural inducer would show little difference in its effects on dorsal and ventral ectoderm (Servetnick and Grainger, 10 Development 112, 177-88, 1991). Therefore we tested noggin's effects on dorsal and ventral ectoderm from the early gastrula. No difference in N-CAM expression is detected (Fig. 5, lanes 4,6), while the ventral animal caps treated with noggin show a greater amount of muscle actin expression (presumably through dorsalization of 15 tissues that received a low-grade mesoderm induction). Activin treated dorsal caps show induction of roughly the same level of muscle actin expression (lane 5) as the ventral noggin treated caps, however, activin treatment did not induce detectable neural specific transcripts (lanes 3,5). This indicates that muscle tissue induced at 20 this stage is not sufficient to secondarily induce neural tissue, and that noggin must be present to induce neural tissue.

We conclude that there is no dorsal-ventral difference in noggin mediated neural induction, suggesting that noggin behaves like the robust neural inducing signal of the Spemann organizer, not 25 like the weaker signal from early anterior mesoderm.

### Dose Dependence

To determine what levels of noggin protein are required for neural inducing activity, we carried out a dose response experiment. In addition to determining the doses required for neural induction in animal caps, we have also carried out a dose response of the dorsalization of ventral marginal zones in order to compare the doses required for these two types of inductions. Stage 9 animal caps or St. 10.5 VMZ were treated with purified human noggin, and N-CAM and  $\beta$ -tubulin were used to assay neural induction, while muscle actin was used as a marker of dorsal mesoderm. This experiment shows that neural induction occurs at a dose of  $1\mu\text{g/ml}$ , which is a twenty fold higher dose than required for dorsalization of VMZ [Fig. 5]. There are several observations that may account for the apparently high dose requirement. First, to get a maximal neural response from dorsal mesoderm, the tissues must be left in contact through most of neurulation (Sharpe and Gurdon, Development 109, 765-74, 1990); in contrast, the animal caps treated with noggin close up rapidly, this inhibits factor access, and consequently they receive only a brief effective dose. Second, it is likely that noggin is not the only neural inducer active in the embryo; it has been shown in a variety of amphibians that the somites (Hemmati-Brivanlou, et al., Science 250, 800-802, 1990; Jones and Woodland, Development 107, 785-91, 1989) and the neural plate have neural inducing activity (Hamburger, The Heritage of Experimental Embryology: Hans Spemann and the Organizer, 1988; Servetnick and Grainger, Dev Biol 147, 73-82, 1991) and noggin transcripts are not detected there. Thus it is plausible that noggin is one of several neural-inducing activities. In this connection it is worth noting that

noggin is equally potent in inducing neural tissue in ventral marginal zones as in dorsalizing them to generate muscle. Numerous other experiments (see Fig. 5) show that induction of a similar amount of muscle at this stage by activin does not result in neural induction.

- 5 Fourth, it may be that only a small fraction of the purified protein is active, and that the experiment overestimates the amount of protein needed for neural induction. Finally, it is possible that the accessibility of exogenously added soluble noggin is significantly lower than noggin protein being secreted endogenously.

10

### Patterning

- Embryonic neural tissue develops an anteroposterior (A-P) pattern, with various brain structures, eyes, and the spinal cord. It is thought that A-P neural pattern requires the presence of dorsal
- 15 mesoderm, whether it be adjacent to the responding ectoderm in a planar configuration (Dixon and Kintner, Development 106, 749-757, 1989; Doniach, et al., Science 257, 542-5, 1992; Kintner and Melton, Development 99, 311-25, 1987; Ruiz i Altaba, Development 108,595-604, 1990) , or directly beneath it in a vertical
- 20 interaction (Dixon and Kintner, Development 106, 749-757, 1989) (Hemmati-Brivanlou, et al., Science 250, 800-802, 1990; Sharpe and Gurdon, Development 109, 765-74, 1990; Sive, et al., Cell 58, 171-180, 1989) . Both of these types of interactions occur in normal development, and both probably contribute to the resulting
- 25 pattern. To determine if noggin induces patterned neural tissue, and if so, what neural regions are represented, we used *Xenopus otx* as a marker of forebrain and mid brain; *En-2* (Hemmati-Brivanlou, et al., Development 111, 715-724, 1991) as a marker of the mid brain-

hind brain boundary, and Krox-20 (Wilkinson, et al., Nature 337, 461-4, 1989) as a marker of the third and fifth rhombomeres of the hind brain in in situ hybridization (Harland, Methods in Cell Biology, 36, 675-685, 1991). Antibodies directed against XlHbox

5 6 (Wright, et al., Development 109, 225-34, 1990) mark posterior hind brain and spinal cord structures. Prior to the use of these markers, we observed the formation of cement glands in noggin treated animal caps. Since cement glands are induced organs of ectodermal origin found anterior to the neural plate, this result

10 suggests that noggin induces anterior structures. In situ hybridization confirms this by showing the presence of a cement gland specific transcript, XAG-1 (Sive, et al., Cell 58, 171-180, 1989) in noggin treated animal caps, but not in control treated animal caps[Fig. 7.]. In situ hybridization with the region specific

15 neural markers[Fig. 7.] show that noggin induces forebrain type tissue as seen by the expression of otx in noggin treated animal caps. We have not detected En-2, Krox20, or XlHbox, suggesting that these more posterior markers are not induced by noggin.

20 Expression of neural antigens

We have demonstrated that noggin directly induces the expression of neural specific transcripts. A further demonstration is to use antibodies against neural specific antigens to show that the noggin induced tissue is phenotypically neural. To this end, we

25 have treated animal cap tissue with noggin and cultured them to a late stage (St 35) for antibody staining. We have used the 6F11 anti-N-CAM antibody, which stains the entire neural tube of a normal embryo. Noggin treated animal caps express this antigen [Fig. 7.]



while control untreated animal caps do not. This indicates that noggin can induce the production of neural specific proteins in treated animal caps. We have failed to detect the expression of numerous other antigens that are characteristic of various subclasses of differentiated neural cells. These included 2G9, which stains most neural tissue, including peripheral neurons, Tor 24.55, which stains sensory neurons, and Tor 23, which stains a variety of neurons including motor neurons.

10

EXAMPLE 7Production of recombinant human noggin from E. coli and baculovirusMaterials and MethodsGenetic Engineering and Cell Culture

15 A lactose inducible expression plasmid was constructed by replacing the Swa1/Bsm1 region of pRPN40 (Masiakowski et al , J. Neurochem. 57, 1003-1012, 1991 ) with the Swa1/Bsm1 region of the human noggin gene obtained by PCR and spanned by the same restriction sites, resulting in plasmid pRG301. pRG301 is a high  
20 copy number kanamycin resistant plasmid derived from pBR322 with the human noggin gene under the control of the lacUV5 promoter. A plasmid containing the high copy number kanamycin resistant gene was deposited with the Agricultural Research Collection (NRRL), Peoria, Illinois, and bears accession number B-18600. This plasmid  
25 was described in United States Patent Application Serial No. 07/478,338, which is incorporated by reference herein in its entirety. E. coli W3110lacIq cells transformed with pRG301 were grown at 37°C, induced with lactose, harvested by centrifugation,

washed once with 100 mM Tris-HCl, 50 mM EDTA pH 8 and stored frozen, essentially as described (Masiakowski et al, *ibid.* ).

#### Recovery from inclusion bodies

- 5 E. coli cell paste (32 g) was suspended in ten volumes (v/w) of 50 mM TrisHCl-pH 8.0-5 mM EDTA, lysed in a French Press at 8,000 psi and 8°C and centrifuged at 8,000xg for 30 min at 4°C. The pellet containing noggin was suspended in the original volume of 2 M urea-20 mM TrisHCl, pH 8.0 and stirred for 30 min. The suspension was
- 10 centrifuged at 8,000xg at 4°C for 30 min and the pellet consisting mostly of inclusion bodies (IB) was suspended in 20 volumes (v/w) of 6 M guanidine HCl, 50 mM TrisHCl, 1 mM EDTA, 50 mM DTT and stirred for one hour at room temperature. After centrifugation at 8,000xg for 30 min, the supernatant containing 0.45-0.50 g
- 15 denatured and reduced noggin was diafiltered against 10 volumes of 6 M urea-50 mM sodium acetate pH 4.5-1 mM EDTA-0.1 mM DTT using Omega 10,000 MW cut-off membranes. The diafiltrate containing 0.4-0.44 g noggin was loaded at a flow rate of 30 ml/min onto a 2.6 x 10 cm column of S-Sepharose (Pharmacia), equilibrated in 6 M urea-50
- 20 mM sodium acetate-1 mM EDTA-0.1 mM DTT pH 4.5 . The column was first washed with the same buffer and then with a one liter gradient (0-1M NaCl) at a flow rate of 30 ml/min. Fractions containing noggin were identified by gel electrophoresis and pooled. The yield was 0.2-0.25 g noggin.

25

#### Refolding.

The denatured and reduced noggin solution was adjusted to .05-.2 mg/ml protein concentration and brought to 1.5-2.5 M

guanidineHCl-0.1 M TrisHCl pH 8.0-0.1 mM EDTA-0.2-2 mM reduced glutathione-0.02-0.2 mM oxidized glutathione (preferably at a ratio of 10:1 reduced to oxidized glutathione) at 4°C under slow stirring. After 24-72 hours, two refolded noggin isoforms were identified by  
5 RP-HPLC chromatography (Fig. 8). The refolded noggin solution was diafiltered against 20 volumes of 0.05 M sodium acetate pH 4.5, brought to 50 mM potassium phosphate pH 7.2 and stirred slowly at 4°C for 1 hour minimum. Misfolded noggin precipitated and was removed by centrifugation for 30 min at 8,000 xg.

10

Reverse phase HPLC chromatography.

Refolded noggin can be purified by chromatography on a 12 mm C8, 1 x 25 cm Dynamax 300 A column equilibrated in solvent A (0.1% TFA in water). After loading, the column was washed with solvent A  
15 and was developed at a flow rate of 4 ml/min according to the following protocol: (a) 10 min isocratically at 70% of solvent A, 30 % of solvent B (0.1% TFA in acetonitrile); 30 min linear gradient to 60 % solvent B and 40 % solvent A. Correctly refolded noggin elutes earlier at 44%-46% solvent B. The yield was 0.07-0.1 g noggin.

20

Production of human noggin in Baculovirus cell culture.

The SF21 line of *Spodoptera frugiperda* was routinely maintained as cell monolayers in Grace's Insect Cell medium supplemented with lactalbumin hydrolysate and yeastolate (Gibco).  
25 This medium completed with 10% v/v heat-inactivated fetal calf serum (Irvine Scientific) is identified as TMNFH-10. Cells were also cultured in serum-free medium (SF-900-II; Gibco) after adaptation. Suspension cultures in either medium were raised in microcarrier

culture flasks (Bellco) using a stirring speed of 80 rpm. All cultures were maintained at >96 % viability, as judged by trypan blue exclusion.

5 Construction of recombinant baculovirus.

Sequences corresponding to human noggin were excised as a 5'-BamH1—Pst1-3' fragment from an expression plasmid containing the human noggin gene. This fragment was inserted into BamH1—Pst1 digested pVL1393 (Invitrogen). The resulting plasmid, pTR 1009,  
10 has the human noggin sequence immediately downstream of the polyhedrin promoter of Autographa californica Multiple Nuclear Polyhedrosis Virus (AcMNPV), and this promoter-heterologous gene fusion is flanked in turn by recombination targets derived from the AcMNPV polyhedrin region. Recombinant plasmid DNA was purified  
15 by alkaline lysis and CsCl centrifugation. SF21 cells were co-transfected with plasmid and viral DNA by the following method: Plasmid DNA (3 mg) was mixed with 0.5 mg linearized, deleted viral DNA (Baculo Gold™, Pharmingen), and precipitated with ethanol. Dried DNA was then resuspended in water (50 ml), mixed with 1.5ml  
20 Grace's medium, and 30 ml Lipofectin™ cationic liposomes (BRL). The DNA-liposome mixture was vortexed, allowed to stand at room temperature for 15 minutes and added dropwise to a monolayer of SF21 cells (2x10<sup>6</sup> cells/60 mm plate). After incubation at 27°C for four hours, 2 ml TMNFH-10 was added and the culture returned to  
25 incubation for 5 days. Tissue culture medium was harvested and used as a source of virus for plaque isolation.

Recombinant virus was isolated by multiple rounds of plaque purification on SF21 cells. Diluted virus (0.5 ml) was adsorbed to

- cell monolayers ( $2 \times 10^6$  cells/60 mm plate) for a period of one hour at  $27^\circ\text{C}$ , aspirated, and virus plaques were allowed to develop with an overlay of 0.5% agarose in TMNFH-10 medium for a period of 6 days. Virus plaques were picked after microscopic inspection, and  
5 eluted into 2ml SF900-II medium. Virus stocks were amplified by low multiplicity (0.1 pfu/cell) infection. Virus clones expressing noggin were identified by metabolic labeling of infected cultures with  $^{35}\text{S}$ -methionine and  $^{35}\text{S}$ -cysteine and analyzing total labeled protein by polyacrylamide gel electrophoresis and autoradiography.  
10 A labeled protein of the expected apparent Mr of 20,000-30,000 was detected by this method in candidate clones but not in control cultures.

Expression and purification of baculovirus-derived noggin.

- 15 SF21 cells were cultured in suspension flasks to a density of approximately  $1.8 \times 10^6/\text{ml}$  in SF900-II medium. Cultures (500 ml) were collected by centrifugation at  $1000 \times g$  for 10 min and resuspended in 20ml of growth medium containing 5-10 pfu/cell recombinant virus. Virus was allowed to adsorb for 1 hour at room  
20 temperature with gentle mixing. Infected cells were then diluted to their original volume with fresh growth medium, and incubated at  $27^\circ\text{C}$  for 3 days. Cells and debris were subsequently clarified from the growth medium by centrifugation at  $1000 \times g$  for 20 min.

- Cell supernatants were brought to pH 8.0, passed through a  
25 0.45 mm Millipak 60 filter and applied to a Fast S column that had been equilibrated in 25 mM HEPES pH 8.0. The column was washed with the same buffer and developed with a linear NaCl gradient to

remove other medium components. Noggin eluted from this column at 1 M NaCl.

### Results

#### 5 Characterization of human noggin produced in E. coli and in baculovirus

Reverse-phase HPLC chromatography shows that recombinant noggin refolded and purified from E. coli elutes in a single sharp peak, indicating the presence of one predominant isoform (Fig. 9).

- 10 Electrophoresis on 15 % polyacrylamide-SDS-reducing gels shows that noggin from either E. coli or insect cells is better than 95 % pure and migrates in a single band corresponding to a protein of 20-30 kD. Noggin from insect cells shows slightly slower mobility, apparently due to additional mass from N-linked glycosylation at the
- 15 single consensus site (Fig. 10). Treatment with Endo F converts the mobility of insect-produced noggin to that of the bacterially produced protein (data not shown).

- In the absence of reducing agents, noggin produced either in E.coli or in baculovirus behaves as a disulfide-linked oligomeric
- 20 protein (Fig. 10). However, by gel filtration analysis and mass spectroscopy noggin is primarily a dimeric protein (data not shown).

- Circular dichroism studies show that recombinant noggin refolded and purified from E. coli as well as noggin purified from insect cells have very similar conformations (Fig. 11). Secondary
- 25 structure determined by this method indicates that noggin consists of 48% alpha-helix, 0 % beta-structure, and 52 % random coil.

Biological Activity of human noggin produced in E. coli and in baculovirus

Biological activity of human noggin produced in E. coli or in baculovirus was determined by assay of muscle actin expression in the ventral marginal zone assay, as described supra. Results shown in Fig. 12 indicate a positive dose response for induction of muscle actin mRNA in VMZ exposed to either bacterially produced human noggin, or baculovirus produced human noggin.

10 EXAMPLE 8

Production and characterization of rat monoclonal antibody RP57-16 reactive with human noggin.

15 Materials and Methods

Production of antibody

RP57-16 rat monoclonal antibody reactive with recombinant human and Xenopus noggin was produced by the immunization of a female Lewis rat with four 35 µg injections of purified recombinant human noggin (produced in E. coli) over a two month period. For the initial immunization, the protein was injected in the rear foot pad in Freund's complete adjuvant. Subsequent injections were given in the same foot pad in Freund's incomplete adjuvant.

25 The rat was euthanized 3 days after the fourth injection.

Lymph node cells from the immunized rat were mixed with SP2/0-E.O. mouse myeloma cells at a ratio of 2:1. After centrifugation, the cell mixture was resuspended in 0.25 ml of 42% (w/v) PEG 3350 (Baker) in phosphate-buffer-saline with 10% (v/v)

dimethylsulfoxide (Sigma) for a total of 3 minutes in a 37°C water bath. Cells were plated at a density of  $5 \times 10^4$  lymphocytes per well in 96-well plates (Falcon 3072) in DMEM/F-12 (Mediatech, Inc.) containing 10% FBS (supplemented with streptomycin, penicillin, pyruvate, and glutamine) and HMGIT ( $1.6 \times 10^{-3}$  M thymidine,  $4.0 \times 10^{-4}$  methotrexate,  $1.3 \times 10^{-3}$  sodium bicarbonate and  $1.0 \times 10^{-2}$  hypoxanthine). After 10 days in culture, supernatants were harvested and assayed for antibody activity against recombinant human noggin by indirect ELISA. Supernatant from COS-M5 cells transfected with the plasmid containing the human noggin gene was air dried overnight in Probind 96-well assay plates (Falcon 3915). Non-specific binding was eliminated by 2 hour incubation at ambient temperature with PBS/1% BSA (Sigma). Plates were washed 2 times with PBS/0.02 % Tween 20. Culture supernatants were then added and incubated at ambient temperature for 1 hour. Plates were washed 4 times with PBS/0.02 % Tween 20. Secondary antibody, Goat anti-Rat IgG (H+L) alkaline phosphatase conjugate (Caltag) diluted 1:2000 in PBS/1% BSA was added to each well and the plates incubated at ambient temperature for 1 hour. Plates were again washed 4 times with PBS/0.02 % Tween 20. Antibody binding was visualized by 1 hour incubation at ambient temperature in the dark with pNPP (p-nitrophenyl phosphate, Sigma) 1 mg/ml in diethanolamine buffer, pH 9.8. The reaction was stopped by the addition of an equal volume of 100 mM EDTA. Absorbance was read at 405 nm on a Thermomax Microplate Reader (Molecular Devices). A reaction was considered positive if the absorbance was 2 times that of the negative control (diluent alone followed by secondary antibody and substrate).



Positive clones were expanded and culture supernatant containing monoclonal antibody was collected for specificity analysis.

RP57-16 was cloned in soft agar. Cloned hybrid cells were expanded in DMEM/F-12 (Mediatech, Inc.) containing 10% FBS (supplemented with streptomycin, penicillin, pyruvate, and glutamine). Supernatant containing antibody was aliquoted and stored at -70°C until use.

#### Specificity Analysis

##### 10 ELISA

100 ng of purified recombinant human noggin, Xenopus noggin, BDNF, NT-3, and NT4 protein was individually passively adsorbed to Probind 96-well assay plates by overnight incubation at 4°C in 50 mM bicarbonate buffer, pH 9.6. BDNF, NT-3 and NT-4 were used to assess non-specific binding of rat monoclonal antibody RP57-16. Supernatants from COS-M5 cells transfected with either the plasmid containing the human noggin gene or the plasmid containing the flg C-terminal tagged Xenopus noggin gene were air dried to Probind 96-well plates overnight. Non-specific binding was eliminated by 2 hour incubation at ambient temperature with PBS/1% BSA (Sigma). Plates were washed 2 times with PBS/0.02 % Tween 20. Undiluted RP57-16 was added and incubated at ambient temperature for 1 hour. Plates were washed 4 times with PBS/0.02 % Tween 20. Secondary antibody, Goat anti-Rat IgG (H+L) alkaline phosphatase conjugate(Caltag) diluted 1:2000 in PBS/1% BSA was added to each well and the plates incubated at ambient temperature for 1 hour. Plates were again washed 4 times with PBS/0.02 % Tween 20. Antibody binding was visualized by 1 hour incubation at ambient

temperature in the dark with pNPP (p-nitrophenyl phosphate, Sigma) 1 mg/ml in diethanolamine buffer, pH 9.8. The reaction was stopped by the addition of an equal volume of 100 mM EDTA. Absorbance was read at 405 nm on a Thermomax Microplate Reader (Molecular Devices). A reaction was considered positive if the absorbance was 2 times that of the negative control (diluent alone followed by secondary antibody and substrate).

#### Electrophoresis and Western Blotting

10 Rat monoclonal antibody RP57-16 was also analyzed by Western blotting. 50 ng of recombinant human noggin, non-reduced and reduced, were electrophoresed on 12.5% SDS-polyacrylamide gels and electroblotted on nitrocellulose membranes. Membranes were blocked with PBS/1% Casein/0.1% Tween 20, and then  
15 incubated for 2 hours with undiluted RP57-16 culture supernatant. Following 4 washes in PBS/0.02% Tween 20, the membranes were incubated with a 1:5000 dilution of Goat anti-Rat IgG (H+L) horseradish peroxidase conjugate (Pelfreeze) in PBS/1% BSA/0.1% Tween 20. Membranes were washed 4 times with PBS/0.02% Tween  
20 20. Proteins were visualized with ECL Western Blotting Reagents (Amersham) according to the manufacturer's instructions. Membranes were then exposed to XAR 5 Scientific Imaging film (Kodak) for 5 seconds.

#### 25 RESULTS

Rat monoclonal antibody RP57-16 reacts with both recombinant human and *Xenopus* noggin and with recombinant human

noggin produced in *E. coli*, in insect cells, and in COS-M5 cells. The antibody does not react with the neurotrophins BDNF, NT-3 and NT-4. Western blotting showed that the antibody detects both reduced and non-reduced protein.

5

#### DEPOSIT OF MICROORGANISMS

The following were deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 under the terms of the Budapest Treaty:

			ATCC Accession <u>No.</u>	<u>Date of Deposit</u>
15	phage	hnog $\lambda$ -5	75311	9-23-92
	phage	hnog $\lambda$ -7	75309	9-23-92
	phage	hnog $\lambda$ -9	75310	9-23-92
	phage	hnog $\lambda$ -10	75308	9-23-92
	hybridoma	RP57-16	CRL 11446	8-25-93

20

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

25